Document **AP2** · Appl. No. 09/665,510

08 25 2503 TIZATION

PCT WORLD INTE	LLECTUA Intern	L PROPERTY ORGANIZATION ational Bureau		
INTERNATIONAL APPLICATION PUBLI	SHED I	INDER THE PATENT COOPERATION TREATY (PCT)		
(51) International Patent Classification ⁶ :		(11) International Publication Number: WO 95/04817		
C12N 5/08, A61K 35/14, 39/00	A1	(43) International Publication Date: 16 February 1995 (16.02.95)		
(21) International Application Number: PCT/US94/08672		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB,		
(22) International Filing Date: 1 August 1994 (01.08.94)				
(30) Priority Data: 08/103,401 6 August 1993 (06.08.93)	, 1	GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD).		
(71) Applicant: CYTEL CORPORATION [US/US]; 3525 John Hopkins Court, San Diego, CA 92121 (US).		Published With international search report.		
(72) Inventors: CELIS, Esteban; 13644 Landfair Road, CA 92130 (US). KUBO, Ralph; 12635 Fu	itura Stre	go, et,		
San Diego, CA 92130 (US). SERRA, Hor Carmel Brooks Way, San Diego, CA 92130 (US). Van; 12892 Ralston Circle, San Diego, CA 92130 (WENTWORTH, Peggy; 11361 Lott Point, San 92126 (US).	US). TS. 92130 (U	AI, S).		
(74) Agents: BASTIAN, Kevin, L. et al.; Townsend and Khourie and Crew, Steuart Street Tower, 20th Market Plaza, San Francisco, CA 94105-1492 (n floor, C	and one		
(54) Title: METHODS FOR EX VIVO THERAPY US. TION OF CIL	ING PEP	TIDE-LOADED ANTIGEN PRESENTING CELLS FOR THE ACTIVA-		
(57) Abstract				
Methods for activating cytotoxic T lymphocytes CIL for therapy in vivo. Additionally, a method for k modified in vitro.	(CTL) in	vitro are presented in conjunction with methods for using the activated cific CTL in vivo is presented using antigen presenting cells which were		
				

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU .	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL.	Netherlands
BF	Burkina Paso	HU	Hungary	NO	Norway
BG	Bulgaria	IR	beland	NZ	New Zealand
BJ	Benin	ä	haly	PL	Poland
BR	Brazil	JP	Japan	77	
BY	Belarus	KE	Kenya	RO	Portugal ·
CA	Canada	KG			Romania
CIF	Central African Republic	_	Kyrgystan	RU	Russian Federation
		KP	Democratic People's Republic	SD	Sudan
CG	Congo		of Korea	SE	Sweden
CE	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM .	Cameroon	Ц	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	T.J	Tajikistan
DE	Germany	MC	Monaco	77	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	ŪA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Pinland	ML	Mali	UZ	Uzbekistan
FR	Prance	MN	Mongolia	VN	Viet Nam
GA	Gabon			. 414	* 500 6 10000

1

5

10

15

20

· 25

30

35

40

. .

METHODS FOR EX VIVO THERAPY USING PEPTIDE-LOADED ANTIGEN PRESENTING CELLS FOR THE ACTIVATION OF CTL

BACKGROUND OF THE INVENTION

The present invention relates to compositions and methods for preventing or treating a number of pathological states such as viral diseases and cancer through ex vivo therapy. In particular, it provides methods for inducing cytotoxic T lymphocytes (CTL) using antigen presenting cells (APC) with a peptide of choice bound to selected major histocompatibility complex (MHC) molecules.

cytotoxic T cells, or CD8 cells as they are also known, represent the main line of defense against viral infections. CTLs specifically recognize and kill cells which are infected by a virus. The T cell receptors on the surface of CTLs cannot recognize foreign antigens directly. In contrast to antibodies, antigen must first be presented to the T cell receptors for activation to occur.

The presentation of antigen to T cells is accomplished by the major histocompatibility complex (MHC) molecules. The major histocompatibility complex (MHC) refers to a large genetic locus encoding an extensive family of glycoproteins which play an important role in the immune response. The MHC genes, which are also referred to as the HLA (human leukocyte antigen) complex, are located on chromosome 6 in humans. The molecules encoded by MHC genes are present on cell surfaces and are largely responsible for recognition of tissue transplants as "non-self".

MHC molecules are classified as either Class I, Class II or class III molecules. Class II MHC molecules are expressed primarily on cells involved in initiating and sustaining immune responses, such as T lymphocytes, B lymphocytes, macrophages, etc. Class II MHC molecules are recognized by helper T lymphocytes and induce proliferation of helper T lymphocytes and amplification of the immune response

```
to the particular immunogenic peptide that is displayed.

to the particular are exoressed on almost all nucleaded that is displayed.
                                            to the particular immunogenic peptide that is displayed.

to the particular immunogenic peptide that is displayed.

to the particular immunogenic peptide that is displayed.

to the particular immunogenic peptide that is displayed.

to the particular immunogenic peptide that is displayed.

to the particular immunogenic peptide that is displayed.

to the particular immunogenic peptide that is displayed.

to the particular immunogenic peptide that is displayed.

To calls and are recognized by common that is displayed.
                                                        Class I MHC molecules are expressed on almost all nucleated to colls and are recognized by and are primarily restricted to cells and are express CD4 and are nucleated to as helper cells express columns.
                                                                   cells and are recognized by CTLS. To cells that serve mainly restricted to represent the recognized by CTLS. To cells are primarily restricted to represent the recognized by CTLS. The represent the recognized by CTLS. The representation of the recogniz
                                                                             as helper cells express CD4 and are primarily restricted to dells, represented colls of molecules whereas conservation class I molecules of cells. Interact with class I molecules of cells of cytotoxic effector cells.
                                                                                    Class II molecules, whereas compressing cells, I molecules.

Class II molecules, whereas interact with che form of a interact with the form of a provided the antique in the form of a provided the cytotoxic effector cells, the antique in the form of a provided the cytotoxic effector complete the form of a provided the cytotoxic effector complete the form of a cytotoxic effector complete the form of a cytotoxic effector complete the cytotoxic effector complete the cytotoxic effector complete the cytotoxic effector complete the cytotoxic effector cytotoxic effe
WO 95/04817
                                                                                                         The CTL recognizes the antigen in the form of a intermediate in the form of a intermediate in the intermediate intermediate intermediate.
                                                                                                                              than the intact foreign antigen is degraded into small per normally be endogenously antigen is degraded into small per normally of the protein antigen is degraded.
                                                                                                                                      normally be endogenously synthesized by the cell, small pertide

normally be endogenously synthesized by the cell, small pertide

gome of the protein antigen is degraded into nentimes

gome of these small nentimes

portion of the cytonlasm.
                                                                                                                     peptide irragment foreign antigen itself.
                                                                                                                                                             fragments in the cytoplasm. Some of these small peptides

fragments in the cytoplasm. Some of these small peptides

compartment and interact with

compartment and interact with

fragments in the cytoplasm. Some of these small peptides

translocate into a pre-Goldi compartment and interact

translocate into a pre-Goldi trate proper folding and

translocate into a pre-Goldi trate proper folding and a pre-Goldi trate proper folding a pre-Goldi trate proper folding and a pre-Goldi trate proper folding a pre-Goldi trate proper folding and a pre-Goldi trate proper folding a pre-Goldi trate proper foldi trate proper fol
                                                                                                                                                                       translocate into a pre-Golgi compartment and interact when the submit R2 microal about the chains to facilitate proper folding and class I heavy the submit R2 microal about the submit R2 microal abo
                                                                                                                                                                                        association with the subunit as then routed to the cell surface is then routed to specific crus.

association with the complex is then routed by specific crus.

peptide-MHC class I potential recognition by specific crus.
                                       5
                                                                                                                                                                                                   Peptide MRC class I complex is then routed to the cell surf.

Peptide MRC class I complex is then routed to the human MRC class I complex is then routed to the human MRC class I complex is then routed by specific MRC c

The peptide MRC class I complex is then routed by specific MRC class I complex is then routed by specific mRC class I complex is then routed to the human MRC class I complex is then routed to the cell surf.
                                                                                                                                                                                 Class I neavy chains to racultate proper rolding to racult
                                                                                                                                                                                                             for expression and potential recognition by specific CTLs. class the human MMC class a nentide binding aroove indicate that a nentide binding aroove.
                                                                                                                                                       fragments in the cytoplasm.
                                                                                                                                                                                                                      I molecule, HIA-A2.1, indicate that a peptide binding of the al and a2 domains of (10 line) of the al and a2 domains of (10 line) of the al. Nature. 329:506 (10 line) of the al. Nature.
                                                                                                                                                                                                                                            is created by the folding of the al., Nature, hoped to raise is created by the (Bjorkman et al., have hoped to raise immunologists have hoped to raise class I heavy chain years, immunologists.
                                                                               10
                                                                                                                                                                                                                                                               For many years, immunologists have retroviruses and viruses, immunize a healthy of the contraction one possible approach is to immunize a healthy one possible approach in the healthy of the h
                                                                                                                                                                                                                                                                         specific cytotoxic cells targeting viruses, individual, and inject cancer cells the criss from this individual, isolate the criss from this individual.
                                                                                                                                                                                                                                                                                    cancer cells isolate diseased person.

cancer cells into the diseased person.
                                                                                                                                                                                                                                                                                                     these cells into work in humans.

these cells to work in humans.

protocol seems to will tried in humans.
                                                                                                                                   15
                                                                                                                                                                                                                                                                                                               Protocol seems to work in inbred mouse strains, but it has not this approach to work in humans. For this approach that of theen successfully tried donor must be identical to that the hard the hardour hard approach to work in humans.
                                                                                                                                                                                                                                                                                                                          been successfully tried in humans. For this approach to work that of the donor must be identical to that of the much haplotype of the important because the crus of the the recipient.
                                                                                                                                                                                                                                                                                              ingly qualify into the diseased person.
                                                                                                                                                                                                                                                                                                                                  the NHC haplotype of the donor must be identical to that of the the CTLS of the important because hound to one of the recipient. Only interact with nentides hound to the the recipient can only interact.
                                                                                                                                                                                                                                                                                                                                             the recipient. This is important because the crus of the the important because bound to one of the peptides bound individual interact with peptides in the individual recipient can only I molecules present in the individual three to six class I molecules
                                                                                                                                                                                     20
                                                                                                                                                                                                                                                                                                                                                       recipient can only interact with peptides bound to one of individual.

I molecules present in the individual.

I molecules present class I molecules with all class I molecules to six class violently with all class I molecules to six react violently with all class I molecules.
                                                                                                                                                                                                                                                                                                                                                                 three to six react those expressed in the individual from whom second, are different from those expressed in the individual from the second.
                                                                                                                                                                                                                                                                                                                                                                          Second, CTLS react violently with all class I molecules which in the individual from whom the individual from the individual tree are different from obtained. regardless of what peptides the the cos cells are obtained.
                                                                                                                                                                                                                                                                                                                                                                                      are different from those expressed in the individual from who the individual from who the individual from who individual from who individual from the individual from who individual from who is the individual from what people is the individual from who is the individual from who is the individual from what people is the individual from what is the individual from the individual from what is the individual from the individu
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   regardices of what here underlying the underlying
                                                                                                                                                                                                                                     25
                                                                                                                                                                                                                                                                                                                                                                                                         Class I molecules contain. This reactivity is the unde transplanted organs.
                                                                                                                                                                                                                                                                                     30
                                                                                                                                                                                                                                                                                                                                                                                                    Class I molecules contain.
                                                                                                                                                                                                                                                                                                                                      35
```

```
Because it is difficult to find two unrelated
                                                       Because it is difficult to find two unrelated

Because it is difficult to find two unrelated

I molecules, annerach

anneraches the non-specific annerach

persons with exactly the take the non-specific annerach

therapentic anneraches take the
                                                                     persons with exactly the same class I molecules, some in vitro take the non-specific approaches take the incubating them in vitro therapeutic approaches cells by incubating therapeutic existing cos cells by incubating them.
                                                                                  therapeutic approaches take the non-specific approach of vitro with incubating them in vitro this protocol therapeutic approaches cells by incubating this protocol However. this protocol However. this protocol However. The protocol approaches take the non-specific approach of vitro with the non-specific approach the non-specific approach the non-specific approaches the no
                                                                                            "boosting" existing CD8 cells by incubating them in vitro wi however, this protocol however, this protocol however, this protocol however, this protocol however, the factor for T cells. (tumor infiltrating infiltr
                                                                                                                     (known as LAK cell therapy or TTL (tumor infiltrating those system is therapy) will only allow the immune system is the immune system as the immune system as the immune system are already activated.
                                                                                                             IL-2, a growth factor for T cells. However, this protection of the approximation of the approximation as LAK cell therapy only allow the expansion of the approximation as LAK therapy will only allow the approximation of the approximation of
WO 95/04817
                                                                                                                                 lymphocytes therapy) will only allow the expansion of those immune system is the immune of the TI..?

Lymphocytes are already activated. As the most of the TI..?

CTLS which are for one reason or another.
                                                                                                                                           crus which are already activated. As the immune system is nost of the IL-2 always active for will be irrelevant for the burbose of always active cells will be irrelevant.
                                                                                                                                                          always active for one reason or another, most of the of the purpose of the purpos
                                                                                                                                                                      stimulated cells will be irrelevant for the purpose of accumented it has not been documented in fact, it has not been the desire combatting the disease.
                                                                                                                                                                                combatting the disease. In fact, it has not been documented it has not been documented it has not been documented and ithe desired and cells with the desired and combatting the disease. In fact, it has not been documented it has not been
                                                                                                                                                                                                                                                                                                                                             and the side effects are often severe. Wechenisms the side of the and alimination of tumor of tumor and alimination of tumors.
                                                                                                                                                                                                                                P. 1991. Adoptive T cell therapy of tumors: Mechanisms Tumor of tumor of tumor Tumor 1992.

P. 1991. Adoptive Tecognition and Melief. C. 1992.

Operative in Immunology 49:281.

Advances in Immunology Agination and Melief.
                                                                                                                                                                                                       specificity. The benefits of JAK cell therapy as the side effects are often severe. The benefits of JAK cell therapy as are often severe. The benefits of JAK cell therapy as are often severe.
                                                   5
                                                                                                                                                                                                                                                        Advances in Immunology 49:281. Melief, C. I lymphocytes.

Melief, C. I lymphocytes.

Melief, C. I lymphocytes.

Melief, C. I lymphocytes.

Melief, C. V. Watanabe. I

Melief, C. V. Wat
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      S. K. Watanabe, J. Watanabe, J.
                                                                                                                                                                                                                                            operative in the recognition and Melief, c.

Advances

Advances

Advances
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       Restoration of
                                                                                                                                                                                                                                                                                           Goodrich, C. Li, M. Agha, P. Greenberg. by the adoptive in immunodeficient humans by the property in clones. Science 257:238.
                                                                                                                                                                                                     specificity.
                                                                                                20
                                                                                                                                                                                                                                                                                                                                                                                                                                         of such sclence 251:238. ). of such treatment of such the preferred approach trie and other infortions are preferred approach trie and other infortions.
                                                                                                                                                                                                                                                                                   Viral immunity in immunodericient numans by the a science 251:238.
                                                                                                                                                                                                                                                                                                                              The preferred approach for the treatment of such infectious and other recognizing hepatitis and cris recognizing diseases would be to activate only those cris recognizing
                                                                                                                                                                                                                                                                     Adv. Cancer Research 58:14.
                                                                                                                                                                                                                                                                                                                                           diseased cells.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      to activate only those cills recognizing in arthrovic while various procedures have been applied in while various procedures have hear metatrowing the market was an arrangement of the same and are same areas and areas and areas are arrangements.
                                                                                                                                                                                                                                                                                                                                                                 diseased cells. While various procedures have been applied in these diseases, few few reported.

These cells have been reported.
                                                                                                                                                            15
                                                                                                                                                                                                                                                                                                                                                                                these diseases, few if any successful attempts using cytotoxic would be the preferable means of treating the types of disease notes of the preferable means of the types of types of the types of the types of the types of types
                                                                                                                                                                                                                                                                                                                                                                                        T cells have been reported. Ex vivo activation of cris would the types of disease noted treating the types have been available procedures have been availab
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               preferable means of treating the types of disease noted to available to have been available procedures have been diseases.

Preferable means of treating the types of diseases.

A constant of the types of disease available to disease available to disease available procedures have these diseases.
                                                                                                                                                                                                                                                                                                                                                                                                                 above. However, no reliable procedures have been available diseases.

Rowever, no reliable procedures have been available at these and other around other around activate addresses these and other around addresses these and other around addresses these are around a procedure and other around addresses these are around a procedure and other around a procedure and a procedure around a procedure and a procedure around a procedure ar
                                                                                                                                                                                                                          20
                                                                                                                                                                                                                                                                                                                                                                                                                             specifically activate crus associated with these diseases.

The present invention addresses these and other problems.
                                                                                                                                                                                                                                                                                                                                                             diseased cells.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     SUMMARY OF THE INVENTION

SUMMARY OF THE INVENTION

This invention is directed to methods of activating

This invention is directed to methods of many

This invention is directed to methods of activating
                                                                                                                                                                                                                                                                                 25
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         cytotoxic T cells (CD8 cells) in vitro or in vivo. The bound of activating CD8 cells on anticen presenting methods of activating CD8 molecules on anticen peptides from class I MRC molecules on anticen peptides
                                                                                                                                                                                                                                                                                                                                                                                                                                                                              This invention is directed to methods of a vivo.

This invention is directed to methods of a vivo.

Aieconia of antivation on a nalle commortee.

Cytotoxic T cells on one nalle commortee.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     methods of activating Lass I MHC molecules on antigen presenting peptides from class I
                                                                                                                                                                                                                                                                                                                                                 30
                                                                                                                                                                                                                                                                                                                                                                                                        35
```

4

cells using a mild acid treatment; associating selected immunogenic peptides with the class I MHC molecule on the antigen presenting cell; and incubating the antigen presenting cells with the cytotoxic T cells, thereby producing activated cytotoxic T cells. The methods of the present invention are capable of generating empty MHC class I molecules on antigen presenting cells and in turn inducing CTL and affecting killing of class I matched cells.

5

The antigen presenting cells having empty MHC class 10 I molecules on their surface are capable of inducing cytotoxic T cells which are useful in the treatment of chronic infectious diseases and cancer. Specifically, this invention provides methods of producing empty MHC class I molecules on antigen presenting cells, loading those empty MHC class I 15 molecules with selected immunogenic peptides, activating cytotoxic T cells which are specific for killing specific antigen targets. This invention has broad therapeutic application in the treatment of cancers, certain immune diseases and viral diseases. As such the method may further 20 comprise: separating activated CTLs from the antigen presenting cells having the empty MHC class I molecule on its surface; suspending the activated CTLs in an acceptable carrier or excipient as a pharmaceutical composition; and administering the pharmaceutical composition to a patient 25 having the disease.

10

15

20

25

30

35

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the effects of β_2 microglobulin and exogenous 941.01 (HB_c 18-27) peptide on MHC class I molecules from acid stripped and loaded PHA blasts

Figure 2 shows CTL induction using GC43 A2.1 responders and autologous acid-stripped PBMCs or PHA blasts loaded with the 777.03 ($HB_{\rm B}$ 20-28); 924.07 ($HB_{\rm C}$ 18-27); 927.32 ($HB_{\rm D}$ 61-69) peptide pool.

Figure 3 shows CTL induction using X351 or X355 A2.1 responders and autologous acid stripped PBMCs or PHA blasts as stimulators after loading with the 1044.04 (PAP 135-143);

1044.05 (PSA 166-175) 1044.06 (PSA 118-128) peptide pool.

Figure 4 shows CTL induction using GC49 A2.1 responders and Autologous Acid stripped PHA blasts as stimulators after loading with 939.03 (PSA 49-57) peptide.

Figure 5 shows CTL induction using GC66 Al responders and autologous acid stripped PBMCs as stimulators after loading of peptide 958.01 (MAGE 1:161-169).

Figure 6 shows CTL induction using GC 30, HLA A1 responders and autologous cold temperature incubated SAC-I activated PBMC $_{\rm B}$ as stimulators after loading with 1044.07 MAGE-3 (161-169) peptides.

Figure 7 shows a comparison of different methods to load peptides onto SAC-I activated PBMCs as APCs. A pool of MAGE-3 HLA A1 binding peptides (1044.07:161-167 and 1044.01:8-17) were tested with donor GC 164. 7 A-acid strip; 7 B- cold temperature incubation; 7 C- room temperature, no preincubation or acid strip with 4 hour peptide loading only; 7 D- room temperature, no acid stripping with addition of soluble peptide to the culture.

Figure 8 shows the result of the CTL induction assay using selected MAGE peptides.

Figure 9 shows the result of the CTL induction assay using selected HIV peptides.

Figure 10 shows the result of the CTL induction assay using selected HCV peptides.

Figure 11 shows the retuls of the CTL induction assay using selected HBV peptides.

```
DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "peptide" is used interchangeably are important and it is used interchangeably are important.
                                                                                        The term "peptide" is used interchangeably with a designate a used interchangeably to designate a one connected one in the present specification connected one two ically I-amino acids.
                                                                                                           "oligopeptide" in the present specification to designate a connected one to connected one to alpha-amino acids, connected the alpha-amino typically bonds between the alpha-amino series of residues, by pentide bonds between the other typically by pentide bonds by the other typically by pentide bonds by the other typically by pentide bonds by the other typically by the
                                                                                                                        series of residues, by peptide bonds between the alpha-amino acids.

series of typically by peptide bonds acids.

the other typically of adjacent amino acids.
WO 95104817
                                                                                                                                                                        An wimmunogenic peptide will sa peptide which the peptide will a comprise an allele and he capable of inducing a comprise an allele and he capable of inducing a comprise and he capable
                                                                                                                                                                                   comprises an allele-specific motif such that the peptide will of inducing a cru response.

comprises an allele and be capable of inducing to an bind the MKC allele and he capable of binding to an bind the MKC allele and he capable of binding to an bind the MKC allele and he capable of binding to an bind the mannagenic pentides are capable.
                                                                                                                                           and carbonyl groups of adjacent amino acids.
                                                                                                                                                                                                      bind the MHC allele and be capable of inducing a cytotoxi

Thus; appropriate class I MHC molecule and inducina
                                                                                                                                                                                                                      Thus, immunogenic peptides are capable of binding to an inducing a cytotoxic rimmunogenic peptides are capable and inducing the immunogenic antigen from which the immunogenic appropriate class I MHC molecule antigen from which the immunogenic appropriate class instruments antigen from which the immunogenic peptides are capable of binding to an inducing a cytotoxic rimmunogenic peptides are capable of binding to an inducing a cytotoxic rimmunogenic peptides are capable of binding a cytotoxic rimmunogenic peptides and inducing a cytotoxic rimmunogenic peptides and inducing a cytotoxic rimmunogenic peptides and inducing a cytotoxic rimmunogenic peptides are capable of peptides and inducing a cytotoxic rimmunogenic peptides are capable of peptides and inducing a cytotoxic rimmunogenic peptides are capable of peptides and inducing a cytotoxic rimmunogenic peptides are capable of peptides and inducing a cytotoxic rimmunogenic peptides are capable of peptides are capable of peptides are capable of peptides and cytotoxic rimmunogenic peptides are capable of peptides and cytotoxic rimmunogenic peptides are capable of peptides and cytotoxic rimmunogenic peptides are capable of peptides are capable of peptides and cytotoxic rimmunogenic peptides are capable of peptides ar
                                                                                                                                                                                                                                         appropriate class I MMC molecule and inducing a cytotoxic T immunogenic appropriate class I MMC molecule and inducing the immunogenic cell response against the antigen from which the immunogenic cell response against the antigen from which the immunogenic cell response against the antigen from which the immunogenic cell response against the antigen from which the immunogenic cell response against the antigen from which the immunogenic cell response against the antigen from which the immunogenic cell response against the antigen from which the immunogenic cell response against the antigen from which the immunogenic cell response against the antigen from which the immunogenic cell response against the antigen from which the immunogenic cell response against the antigen from which the immunogenic cell response against the antigen from which the immunogenic cell response against the antigen from which the immunogenic cell response against the antigen from the cell response against the cell respon
                                                                                                                                                                                                                                                                                                                                                                                                                            derived. "residue" refers to an amino acid or acid
                                                                                                                                                                                                                                                                                    The term "residue" refers to an amino acid or amide bond an incorporated in an oligopeptide by an amide bond acid mimetic incorporated.
                                                                5
                                                                                                                                                                                                                                                                                                                                 The present invention relates to methods of using extraction relates to various diseases using extraction comprise to various the invention comprise to methods of the invention comprise to various diseases.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                            The present invention relates to methods of
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             Immune response to various diseases using ex

The general approach of the color of the general approach of the color of th
                                                                                                                                                                                                                                                                                                                                                               vivo therapy. The general approach of the invention comprises into the invention of the cells (PBMCS) into the invention of the cells into the invention of peripheral blood mononuclear neotide into the isolation of peripheral immunodenic neotide into the isolation of peripheral immunodenic neotide into the isolation of peripheral immunodenic neotide into the invention and into the invention and into the invention and invention approach of the invention and invention approach of the invention and invention and invention are inventional and invention are inventional are
                                                                                                                                                                                                                                                         pertide is derived.
                                                                                                                           20
                                                                                                                                                                                                                                                                                                                                                                               isolation of peripheral blood mononuclear cells into the surface immunogenic peptide into the surface immunogenic peptide into the surface immunogenic peptide in the surface patient, patient, pockets of MRC class I molecules on the surface binding pockets of MRC class I molecules on the surface peptide in the surface immunogenic peptide into the surface immunogenic peptide immuno
                                                                                                                                                                                                                                                                                                                                                                                            Patient, loading a desired immunogenic Peptide into the surface of the Apra with incubating the Apra with binding pockets of MRC class [Apcs].
                                                                                                                                                                                                                                                                                                            or amide bond mimetic.
                                                                                                                                                                                                                                                                                                                                                                                                               binding pockets of MMC class I molecules on the Apcs with incubating the Apcs of CI incubating proliferation of CI antigen presenting the sample to induce proliferation of calls antigen precursor CTLS in the sample to prec
                                                                                                                                                                                                                                                                                                                                                                                                                            antigen presenting the sample to induce proliferation of the proliferation 
                                                                                                                                                                                                                                                                                                                                                                                                                                            precursor crus in the sample to induce proliferation or constant the crus in the sample to indentify to identify to identify the crus introduced in the crus introduced in the crus introduced in the sample to indentify the introduced in the peptide, and by expanding their numbers introduced in the peptide, and by expanding their numbers in the sample to induce proliferation or crus induced proliferation or crus in the sample to induce proliferation or crus induced 
                                                                                                                                                                                                                                                                                                                                                                                                                                                            recognizing the pertide, and using the crus to identify numbers introduce and using their numbers introduce the activated crus into the patient.
                                                                                                                                                                                                              15
                                                                                                                                                                                                                                                                                                                                                         vivo therapy.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             ated CILS into the patient. ent invention depend in me procedures of the present invention of anirones roomen's and her amin at the determination of anirones roomen's anirones.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       The procedures of the present invention depend in invention depend by crist one approach one approach one approach one approach the determination target infected cells.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             Part upon the determination of epitopes recognized by cris one approach to infected cells.

Part upon the determination target the identification of these epitopes is the identification of these identification of these identification of these epitopes is the identification of these epitopes.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                               an activated one into the patient.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          capable of eliminating target infected cells. one approach the identification of these motifs associated with a narticular identification of these motifs associated with a narticular identification of peortide motifs associated with a narticular identification of peortide motifs associated with a narticular identification of the identification of the
                                                                                                                                                                                                                                                                                         20
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          identification of these epitopes is the identification of these epitopes associated with a particular me mic class allele subtypes.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           The Mic class
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     I antigens are expressed at the expression of HIA-C is

B antigens are expressed the expression of HIA-C is

emal densities.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        allele-specific peptide motils associated with a disease for human class in the article subtypes.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     disease for human class I MRC allele subtypes. loci.

The Rank and C at anny

disease for human class by the Rank and c at anny

antiquene are armressed at the coll surface at anny

Rankinene are armressed at the coll
                                                                                                                                                                                                                                                                                                                                                                 25
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        B antigens are expressed at the cell surface at approximate an approximate antigens are expressed the expression of Hid-C is whereas the expression of In-fala in-fala
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     equal densities, whereas the expression of Hua-C 1s lower).

equal densities, whereas the expression as 10-fold lower a number of alleles. A large number of alleles a large number of these loci have a number of these local have a number of the l
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   significantly lower a number of alleles.

Significantly lower a number of alleles.

A large number of alleles.
                                                                                                                                                                                                                                                                                                                                                                                                                                               30
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               35
```

```
cells with defined MRC molecules, particularly MRC class I molecules, particularly made readily available.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         These cells can
                                                         With target diseases.

The allele-specific motifs are then used to define To the martines of the allele-specific motifs are then used to define To the martines of the allele-specific motifs are then used to define To the then used to the the then used to the theory to the then used to the theory to the theor
                                                 cells with derined MHC Morecutes, harriculari
                                                                                         The allele-specific motifs are then used to those allele-specific motifs are then used to those particularly which any desired antigen, cancers, for which cell epitopes from any viral diseases or cancers, associated with numan viral diseases.
                                                                                                cell epitopes from any desired antigen, particularly which is known.

cell epitopes from any viral diseases or cancers, is known.

associated with numan wiral notential antigen targets is known.
WO 95/04817
                                                                                                            associated with human viral diseases or cancers, for which the role is known.

This general approach is described in detail in conending and anino general approach is described in detail in conending anino general approach is described in detail in conending anino general approach is described in detail in conending anino general approach is described in detail in conending anino general approach is described in detail in conending anino general approach is described in detail in conending anino general approach is described in detail in conending anino general approach is described in detail in conending anino general approach is described in detail in conending anino general approach is described in detail in conending anino general approach is described in detail in conending anino general approach is described in detail in conending anino general approach is described in detail in conending anino general approach is described in detail in conending anino general approach is described in detail in conending anino general approach is described in detail in cone general approach is described in detail in
                                                                                                                       amino acid sequence of the potential antigen in copending and in detail in copending and in detail in copending and in described in detail in 071926.666 and or of the potential antigen in copending and in detail in detail in detail in detail in copending and in detail in det
                                                                       associated with target diseases.
                                                                                                                                 commonly assigned applications U.S.S.N. 07/926,666 and u.S.S.N. 08/027,146, which are incorporated herein by
                                                                                                                                                                                                                                                                     Potential epitopes on a number of target proteins
                                                                                                                                                                                    can be identified in this manner. Examples of suitable (PSA), hepatitis B antigen (PSA), hepatitis B antigen (PSA), hepatitis B (PSA), hepatitis B
                                                                                                                                                                           potential epitopes on a number or target protein herati
                                                                                                                                                                                               antigens include prostate specific antigens (PSA), hepatitific antigens (PSA), hepatit
                                             5
                                                                                                                                                                                                       core surface and polymerase antigens immunodeficiency virus (HIV)

the partitis c antigens (e.a. MAGE-1).
                                                                                                                                                                                                                  nepatitis c antigens, Epstein-Barr virus antigens, melanoma immunodeficiency virus (HIV)

nepatitis (e.g., MACE-1), virus (HPV) antigens, antigens numan papilloma virus antigens, numan papilloma antigens, numan papilloma virus (HPV)
                                                                                                                                                                                                                                       antigens, human papilloma virus (HPV) antigens, human papilloma virus simplex virus (HSV).

antigens, human papilloma virus cEA, p 53-breast ovary.

cytomegalovirus (C-Erb B, CEA, p 53-breast ovary).
                                                                                                                                                               reference.
                                                                                                                                                                                                                            antigens, human papilloma virus (HPV) antigens, antigens, human papilloma virus aimpleus virus (HPV) antigens, antigens, lower, however aimpleus virus (HPV)
                                                                                    10
                                                                                                                                                                                                                                                   cycomegalovirus (CMV), herpes simplex virus (HSV), and cycomegalovirus (CMV), herpes cRA, p 53-breast oncodene products (c-Erb B2, typically involve isolation oncodene rhese approaches
                                                                                                                                                                                                                                                                                                                                                             products (c-Erd B2, cra, b 23-preast ovary).

These approaches typically involve isolation of the grant and semicandary the grant and semicandary.
                                                                                                                                                                                                                                                                     These approaches typically involve isolation of the molecule and sequencing the number of the pertials from a particular relevant motif.

Pertials from a particular relevant motif.
                                                                                                                                                                                                                                                                            Peptides to determine the relevant method a method for peptides 242.1068 (1088) first described a method for peptides 242.1068 (1088)
                                                                                                                                                                                                                                                                                        Science, of bound pertides from Nature. 351:290 (1991) and his coworkers
                                                                                                                                       15
                                                                                                                                                                                                                                                                                                             elution of bound pertides from Mic. Subsequently, Ramme and his coworkers an annroach to characterize naturally nrocess and his coworkers an annroach to characterize naturally nrocess and his coworkers.
                                                                                                                                                                                                                                                                                                                      and his coworkers (Falk et al., Nature, naturally processed other investigators other investigators approach to class I molecules.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 Other investigators have
                                                                                                                                                                                                                                                                                                                                           peptides bound to class I molecules acid sequencing of the more fractions by conventional fractions by conventional successfully achieved various HPLC fractions by conventional successfully achieved warlous HPLC fractions by conventional fractional fract
                                                                                                                                                                                                                                                                                                                                                     successfully achieved direct amino acid sequencing of the mon acid sequencing of the mon acid sequencing of the mon acid sequencing of conventional by conventional archieved direct amino fractions by conventional archieved direct amino fractions by conventional archieved direct amino acid sequencing acid sequencing acid sequencing of the mon a
                                                                                                                                                                                      20
                                                                                                                                                                                                                                                                                                                                                                abundant peptides in various HPIC fractions by conventional I and of peptides eluted from B (1991) and of peptides al., Nature. 353:326 (1991) and automated sequencing of peptides al., Nature. 353:326 (Jardetzky, et al., Nature. 353:326)
                                                                                                                                                                                                                                                                                                                                                                         automated sequencing of peptides eluted from B type class I science.

automated sequencing of peptides al., Nature, (Hunt. et al., Science)

automated sequencing et al., Nature, (Hunt. et al., Science)

the A2.1 type by mass spectrometry (Hunt. et al., Science)

the A2.1 type by mass spectrometry (Hunt. et al., Science)
                                                                                                                                                                                                                                                                                                                                  peptides bound to class I molecules.
                                                                                                                                                                                                                                                                                                                                                                                   molecules (Jardetzky, et al., Nature, Hunt, et al., of the characterization of the A2.1 type by mass spectrometry of the characterization of the A2.1 (1992).
                                                                                                                                                                                                                                                                                                                                                                                                     225:1261 (1992). A review of the characterization of molecules is found on MHC class Falk. Immunol. (Rötzschke and Falk (Rötzschke and Falk (Rötzschke and Falk presented by Rötzschke and Falk (Rötzschke and
                                                                                                                                                                                                                                                                                                                                                                                                               naturally processed peptides found on MHC Class I molecules is found in the MHC class I molecule in the MHC cl
                                                                                                                                                                                                                                                                                          30
                                                                                                                                                                                                                                                                                                                                                                                                 225:1261 (1992).
                                                                                                                                                                                                                                                                                                                                                                                                                               Today, 12:447 (1991).
```

10

15

20

25

30

35

Definition of motifs specific for different class I alleles allows the identification of potential peptide epitopes from an antigenic protein whos amino acid sequence Typically, identification of potential peptide epitopes is initially carried out using a computer to scan the amino acid sequence of a desired antigen for the presence of The epitopic sequences are then synthesized. capacity to bind MHC Class molecules is measured in a variety of different ways using, for example, purified class I molecules and radioiodinated peptides and/or cells expressing empty class I molecules by, for instance, immunofluorescent staining and flow microfluorimetry, peptide-dependent class I assembly assays, and inhibition of CTL recognition by peptide competition. Other alternatives described in the literature include inhibition of antigen presentation (Sette, et al., J. Immunol., 141:3893 (1991), in vitro assembly assays (Townsend, et al., Cell, 62:285 (1990), and FACS based assays using mutated ells, such as RMA.S (Melief, et al., Eur. J. Immunol., 21:2963 [1991]).

Next, peptides that test positive in the MHC class I binding assay are assayed for the ability of the peptides to induce specific primary or secondary CTL responses in vitro. For instance, antigen-presenting cells that have been incubated with a peptide can be assayed for the ability to induce CTL responses in responder cell populations. For secondary responses, antigen-presenting cells can be normal cells such as peripheral blood mononuclear cells or dendritic cells (Inaba, et al., J. Exp. Med., 166:182 (1987); Boog, Eur. J. Immunol., 18:219 [1988]).

Alternatively, mutant mammalian cell lines that are deficient in their ability to load class I molecules with internally processed peptides, such as the mouse cell lines RMA-S (Kärre, et al.. Nature, 319:675 (1986); Ljunggren, et al., Eur. J. Immunol., 21:2963-2970 (1991)), and the human somatic T cell hybridoma, T-2 (Cerundolo, et al., Nature, 345:449-452 (1990)) and which have been transfected with the appropriat human class I g nes are conveniently used, when peptide is added to them, to test for the capacity of the

10

15

20

25

30

35.

9

peptide to induce in vitro primary CTL responses. These empty MHC cells are preferable for inducing a primary response since the density of MHC-peptide complexes on the surface of the antigen presenting cell will be greater. Other eukaryotic cell lines which could be used include various insect cell lines such as mosquito larvae (ATCC cell lines CCL 125, 126, 1660, 1591, 6585, 6586), silkworm (ATTC CRL 8851), armyworm (ATCC CRL 1711), moth (ATCC CCL 80) and Drosophila cell lines such as a Schneider cell line that have been transfected with the appropriate human class I MHC allele encoding genes and the human B₂ microglobulin genes.

Once the appropriate epitope is determined, immunogenic peptides comprising the motif required for MHC binding and the epitope recognized by the CTL are synthesized. The immunogenic peptides can be prepared synthetically, or by recombinant DNA technology or isolated from natural sources such as whole viruses or tumors. One of skill will recognize that the immunogenic peptides can be a variety of lengths, either in their neutral (uncharged) forms or in forms which are salts, and either free of modifications such as glycosylation, side chain oxidation, or phosphorylation or containing these modifications, subject to the condition that the modification not destroy the biological activity of the polypeptides as herein described.

Desirably, the peptide will be as small as possible while still maintaining substantially all of the biological activity of the large peptide. When possible, it may be desirable to optimize peptides of the invention to a length of 9 or 10 amino acid residues, commensurate in size with endogenously processed viral peptides or tumor cell peptides that are bound to MHC class I molecules on the cell surface.

Peptides having the desired activity may be modified as necessary to provide certain desired attributes, e.g., improved pharmacological characteristics, while increasing or at least retaining substantially all of the biological activity of the unmodified peptide to bind the desired MHC molecule and activate the appropriate T cell. For instance, the peptides may be subject to various changes, such as

substitutions, either conservative or non-conservative, where such changes might provide for certain advantages in their use, such as improved MHC binding. By conservative substitutions is meant replacing an amino acid residue with another which is biologically and/or chemically similar, e.g., one hydrophobic residue for another, or one polar residue for another. The substitutions include combinations such as Gly, Ala; Val, Ile, Leu, Met; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr. The effect of single amino acid substitutions may also be probed using D-amino acids. Such modifications may be made using well known peptide synthesis procedures.

The peptides of the invention can be prepared in a wide variety of ways. Because of their relatively short size, the peptides can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young, Solid Phase Peptide Synthesis, 2d. ed., Pierce Chemical Co. (1984), supra.

Alternatively, recombinant DNA technology may be employed wherein a nucleotide sequence which encodes an immunogenic peptide of interest is inserted into an expression vector, transformed or transfected into an appropriate host cell and cultivated under conditions suitable for expression. These procedures are generally known in the art, as described generally in Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York (1982), which is incorporated herein by reference. Fusion proteins which comprise one or more peptide sequences of the invention can also be used to present the appropriate T cell epitope.

The immunogenic peptides are then used to activate CTL ex vivo. The ex vivo therapy methods of the present invention and pharmaceutical compositions thereof are useful for treatment of mammals, particularly humans, to treat and/or pr vent viral infection, immune disorders and cancer. Examples of dis ases which can be treated using the ex vivo

10

15

20

25

30

35

therapy methods of the invention include prostate cancer, hepatitis B, hepatitis C, AIDS, renal carcinoma, cervical carcinoma, lymphoma, CMV, condyloma acuminatum breast and ovarian cancer, colon, lung cancer and HSV.

For therapeutic use, therapy should begin at the first sign of viral infection or the detection or surgical removal of tumors or shortly after diagnosis in the case of acute infection. This is followed by boosting levels of CTL at least until symptoms are substantially abated and for a period thereafter. In chronic infection, loading doses followed by boosting doses may be required.

Treatment of an infected individual with the methods of the invention may hasten resolution of the infection in acutely infected individuals. For those individuals susceptible (or predisposed) to developing chronic infection the methods are useful for preventing the evolution from acute to chronic infection. Where the susceptible individuals are identified prior to or during infection, the compositions can be targeted to them, minimizing the need for administration to a larger population.

The methods of the present invention can also be used for the treatment of chronic infection and to stimulate the immune system to eliminate virus-infected cells in carriers.

Ex vivo CTL responses to a particular pathogen (infectious agent or tumor antigen) are induced by incubating in tissue culture the patient's CTL precursor cells (CTLp) together with a source of antigen-presenting cells (APC) loaded with the appropriate immunogenic peptide. After an appropriate incubation time (typically 3-12 weeks) in which the CTLp are activated and mature and expand into effector CTL, the cells are infused back into the patient, where they will destroy their specific target cell (an infected cell or a tumor cell). Infusion of the cells into the patient may include a T cell growth factor such as interleukin 2 (IL-2). In order to optimize the *in vitro* conditions for the generation of specific cytotoxic T cells, the culture of stimulator cells is maintained in an appropriate serum-free

12

medium which may include one or more growth factors such as IL-2, IL-4, IL-7 and IL-12.

5

10

15

20

25

30

35

Peripheral blood lymphocytes are conveniently isolated following simple venipuncture or leukapheresis of normal donors or patients and used as the responder cell sources of CTLp. In one embodiment particularly for secondary CTL responses, the appropriate APC are incubated with 10-100 µM of peptide in serum-free media for 4 hours under appropriate culture conditions. The peptide-loaded APC are then incubated with the responder cell populations in vitro for 7 to 10 days under optimized culture conditions. For primary CTL induction, APC expressing empty MHC would be used to stimulate naive CTLp. In this case the CTL would be stimulated more frequently (1-2 times).

Positive CTL activation can be determined by assaying the cultures for the presence of CTLs that kill radiolabeled target cells, both specific peptide-pulsed targets as well as target cells expressing endogenously processed form of the relevant virus or tumor antigen from which the peptide sequence was derived.

Specificity and MHC restriction of the CTL of a patient can be determined by a number of methods known in the art. For instance, CTL restriction can be determined by testing against different peptide loaded target cells expressing human MHC class I alleles shared with the HLA phonotype of the donor CTL. The peptides that test positive in the MHC binding assays and give rise to specific CTL responses are identified as immunogenic peptides.

As mentioned above, the induction of CTL in vitro requires the specific recognition of peptides that are bound to allele specific MHC class I molecules on APC. The number of specific MHC/peptide complexes per APC determines the level of stimulation of CTL, particularly during the primary immune response. While small amounts of peptide/MHC complexes per cell are sufficient to render a cell susceptible to lysis by CTL, or to stimulate a secondary CTL response, the successful activation of a CTLp during primary response requires a significantly higher number of MHC/peptide complexes.

13

Since mutant cell lines capable of expressing empty MHC do not exist for every human MHC allele, it is advantageous to use a technique to remove endogenous MHC-associated peptides from the surface of APC, followed by loading the resulting empty MHC molecules with the immunogenic The use of non-transformed peptides of interest. (non-tumorigenic), non-infected cells, and preferably, autologous cells of patients as APC is desirable for the design of CTL induction protocols directed towards development of ex vivo CTL therapies. This present invention provides novel methods generating empty class I MHC which can then be loaded with an appropriate immunogenic peptide by stripping the endogenous MHC-associated peptides from the surface of APC or through cold temperature incubation (37°C→26°C) followed by the loading of desired peptides.

10

15

20

25

30

35

A stable MHC class I molecule is a trimeric complex formed of the following elements: 1) a peptide usually of 8 - 10 residues, 2) a transmembrane heavy polymorphic protein chain which bears the peptide-binding site in its α 1 and α 2 domains, and 3) a non-covalently associated non-polymorphic light chain, β_2 microglobulin. Removing the bound peptides and/or dissociating the β_2 microglobulin from the complex renders the MHC class I molecules nonfunctional and unstable, resulting in rapid degradation at 37°C. Almost all MHC class I molecules isolated from PBMCs have endogenous peptides bound to them. Therefore, the first step to prepare APC for primary CTL induction is to remove all endogenous peptides bound to MHC class I molecules on the APC without causing degradation or cell death before exogenous peptides can be added.

Two possible ways to generate free MHC class I molecules include lowering the culture temperature from 37°C to 26°C overnight to allow MHC class I without peptides to be expressed and stripping the endogenous peptides from the cell using a mild acid treatment. The mild acid treatment releases previously bound peptides into the extracellular environment allowing new exogenous peptides to bind to the empty class I molecul s. The overnight cold-temperature incubation at 26°C which may slow the cell's metabolic rate enables expression of

14

stable empty class I molecules which then bind exogenous peptides efficiently. It is also likely that cells not actively synthesizing MHC molecules (.g., resting PBMC) would not produce high amounts of empty surface MHC molecules by the cold temperature procedure.

5

10

15

20

25

30

35

Extraction of the peptides is accomplished by harsh acid stripping using trifluoroacetic acid, pH 2, or acid denaturation of the immunoaffinity purified class I-peptide complexes. These methods are not feasible for CTL induction, since it is important to remove the endogenous peptides while preserving APC viability and an optimal metabolic state which is critical for antigen presentation. Mild acid solutions of pH 3 such as glycine or citrate-phosphate buffers have been used to identify endogenous peptides and to identify tumor associated T cell epitopes (31. Storkus, W., H. Zeh, R. Salter, and M. Lotze. 1993. Identification of T cell epitopes: Rapid isolation of class I-presented peptides from viable cells by mild acid elution [submitted]). The treatment is especially effective, in that only the MHC class I molecules are destabilized (and associated peptides released), while other surface antigens remain intact, including MHC class II molecules. (16. Suguwara, S., T. Abo, and K. 1987. A simple method to eliminate the antigenicity of surface class I MHC molecules from the membrane of viable cells by acid treatment at pH 3. J. Immunol. Meth. 100:83). Most importantly, treatment of cells with the mild acid solutions do not affect the cell's viability or metabolic The mild acid treatment is rapid since the stripping of the endogenous peptides occurs in two minutes at 4°C and the APC is functional after the appropriate peptides are loaded. The technique is utilized herein to make peptidespecific APCs for the generation of primary antigen-specific CTL. The resulting APCs are efficient in inducing peptide-specific CTL.

Typically in a primary response prior to incubation of the APCs with the CTLp to be activated, an amount of antig nic peptide is added to the APCs or stimulator c ll culture, of suffici nt quantity to become loaded onto the

10

15

20

25

30

35

and the second

human Class I molecules to be expressed on the surface of the APCs. In the present invention, a sufficient amount of peptide is an amount that will allow about 200 or more human Class I MHC molecules loaded with peptide to be expressed on the surface of each stimulator cell. Preferably, the stimulator cells are incubated with 5-100µg/ml peptide.

Resting or precursor CTLs are then incubated in culture with the appropriate APCs for a time period sufficient to activate the CTLs. The CTLs are activated in an antigenspecific manner. The ratio of precursor CTLs to APCs may vary from individual to individual and may further depend upon variables such as the amenability of an individual's lymphocytes to culturing conditions and the nature and severity of the disease condition or other condition for which the within-described treatment modality is used. Preferably, however, the CTL:APC (i.e. responder to stimulator) ratio is in the range of about 10:1 to 100:1. The CTL/APC culture may be maintained for as long a time as is necessary to stimulate a therapeutically useable or effective number of CTL.

Activated CTL may be effectively separated from the APC using one of a variety of known methods. For example, monoclonal antibodies specific for the APCs, for the peptides loaded onto the stimulator cells, or for the CTL (or a segment thereof) may be utilized to bind their appropriate complementary ligand. Antibody-tagged cells may then be extracted from the admixture via appropriate means, e.g., via well-known immunoprecipitation or immunoassay methods.

Effective, cytotoxic amounts of the activated CTLs can vary between in vitro and in vivo uses, as well as with the amount and type of cells that are the ultimate target of these killer cells. The amount will also vary depending on the condition of the patient and should be determined via consideration of all appropriate factors by the practitioner. Preferably, however, about 1 X 10⁶ to about 1 X 10¹², more preferably about 1 X 10⁸ to about 1 X 10¹¹, and even more preferably, about 1 X 10⁹ to about 1 X 10¹⁰ activated CTLS are utilized for adult humans, compared to about 5 X 10⁶ - 5 X 10⁷ cells used in mice.

10

15

20

25

30

35

As discussed above, the activated CTLS may be harvested from the cell culture prior to administration of the cells to the individual being treated. It is important to note, however, that unlike other present treatment modalities, the present method uses a cell culture system that does not contain transformed or tumor cells. Therefore, if complete separation of antigen-presenting cells and activated CTLS is not achieved, there is no inherent danger known to be associated with the administration of a small number of stimulator cells, whereas administration of mammalian tumor-promoting cells may be extremely hazardous.

One embodiment of the present invention uses the APC generated by the in vitro techniques of this application for therapy against CTL in vivo. In this embodiment, the APC are a patient's cells (e.g., the peripheral blood cells) which are stripped of their natural antigenic peptides and loaded with a peptide of choice which is conjugated to a toxin (e.g. ricin A chain or pseudomonas toxin). The APCs are then re-introduced into the patient, where they will be bound by the endogenous CTLs that are specific for the antigenic peptide. The coupled toxin will kill the activated CTL that are harmful i.e. those which stimulate transplant rejection after it binds the APC. Such directed CTL killing is broadly useful for treating tissue-transplantation rejection and auto-immune disorders, which are mediated through CTL. The treatment regime will vary depending upon the specific disorder to be treated and the judgement of the treating physician.

Methods of re-introducing cellular components are known in the art and include procedures such as those exemplified in U.S. Patent No. 4,844,893 to Honsik, et al. and U.S. Patent No. 4,690,915 to Rosenberg, which are incorporated herein by reference. For example, administration of activated CTLs via intravenous infusion is appropriate.

The following examples are offered by way of illustration, not by way of limitation.

15

20

25

30 .

35

Example 1

Ex vivo induction of Cytotoxic T Lymphocytes (CTL)

Peripheral blood mononuclear cells (PBMC) are isolated from an HLA-typed patient by either venipuncture or leukapheresis (depending upon the initial amount of CTLp required), and purified by gradient centrifugation using Ficoll-Paque (Pharmacia). Typically, one can obtain one million PBMC for every ml of peripheral blood, or alternatively, a typical leukapheresis procedure can yield up to a total of 1-10 X 10¹⁰ PBMC.

The isolated and purified PBMC are co-cultured with an appropriate number of APC expressing empty MHC molecules, previously incubated ("pulsed") with an appropriate amount of synthetic peptide (containing the HLA binding motif and the sequence of the antigen in question). PBMC are usually incubated at 1-3 X 10⁶ cells/ml in culture medium such as RPMI-1640 (with autologous serum or plasma) or the serum-free medium AIM-V (Gibco).

APC are usually used at concentrations ranging from 1X10⁴ to 1X10⁶ cells/ml, depending on the type of cell used. Possible sources of APC include: autologous PBMCs, SAC-I activated PBMCs, PHA blasts; autologous dendritic cells (DC) which are isolated from PBMC and purified as described (Inaba, et al., J. Exp. Med., 166:182 (1987)); and mutant and genetically engineered mammalian cells such as the mouse RMA-S cell line or the human T2 cell line transfected with the appropriate MHC genes that express "empty" HLA molecules which are syngeneic to the patient's allelic HLA form). APC containing empty HLA molecules are known to be potent inducers of CTL responses, possibly because the peptide can associate more readily with empty MHC molecules than with MHC molecules which are occupied by other peptides (DeBruijn, et al., Eur. J. Immunol., 21:2963-2970 (1991)).

The APC are gamma irradiated with an appropriate dose (using, e.g., radioactive cesium or cobalt) to prevent their proliferation and to facilitate the expansion of the CTLp.

15

20

25

30

35

The mixture cultures, containing PBMC, APC and peptide are kept in an appropriate culture vessel such as plastic T-flasks, gas-permeable plastic bags, or roller bottles, at 37° centigrade in a humid air/CO2 incubator. After the activation phase of the culture, which usually occurs during the first 3-5 days, the resulting effector CTL can be further expanded, by the addition of recombinant growth factors such as interleukin-2 (IL-2), interleukin-4 (IL-4), or interleukin-7 (IL-7) to the cultures. An expansion culture can be kept for an additional 5 to 12 days, depending on the numbers of effector CTL required for a particular patient. addition, expansion cultures may be performed using hollow_ fiber artificial capillary systems (Cellco), where larger numbers of cells (up to 1X1011) can be maintained. In order to obtain the required cell numbers for treatment, it may be necessary to restimulate the cultures 2-4 times with irradiated, autologous, peptide pulsed adherent PBMCs.

Before the cells are infused into the patient, they are tested for activity, viability, toxicity and sterility. The cytotoxic activity of the resulting CTL can be determined by a standard ^{5I}Cr-release assay (Biddison, W.E. 1991, Current Protocols in Immunology, p7,17.1-7.17.5, Ed. J. Coligan et al., J. Wiley and Sons, New York), using target cells that express the appropriate HLA molecule, in the presence and absence of the immunogenic peptide. Viability is determined by the exclusion of trypan blue dye by live cells. Cells are tested for the presence of endotoxin by conventional techniques. Finally, the presence of bacterial or fungal contamination is determined by appropriate microbiological methods (chocolate agar, etc.). Once the cells pass all quality control and safety tests, they are washed and placed in the appropriate infusion solution (Ringer/glucose lactate/human serum albumin) which may include a T-cell growth factor such as IL-2 and infused intravenously into the patient.

10

15

20

Example 2

Preparation of effective HLA allele-specific antigen presenting cells by acid stripping followed by peptide loading.

This example demonstrates the use of cold temperature incubation and acid stripping for generation of empty MHC class I molecules to enable peptide loading method to prepare effective HLA-allele-specific antigen presenting cells (APC) for use in diagnostic or ex vivo therapy applications. The APC in this example were used to sensitize precursor cytotoxic T lymphocytes for the development of antigen-specific cytotoxic cells. This was accomplished using either staphylococcus aureus cowan I SAC I activated PBMC, phytohemagglutinin (PHA) T-cell blasts or peripheral blood mononuclear cells (PBMC) as APC in the HLA-A2.1 and HLA-A1 systems. The results are applicable to other APC and to the other MHC alleles.

Culture Medium. PHA blasts and CTL inductions were done in RPMI 1640 + Hepes + glutamine (Gibco) supplemented with 2 mM L-glutamine (Irvine Scientific), 50 µg/ml gentamicin (Gibco), and 5% heat inactivated pooled human Type AB serum (Gemini Bioproducts) [RPMI/5% HS]. EBV transformed lymphoblastoid cell lines (LCL) were maintained in RPMI 1640 + Hepes + glutamine (BioWhittaker) supplemented with L-glutamine and gentamicin as above and 10% heat inactivated fetal calf serum (Irvine Scientific) [RPMI/10% FCS]. Chromium release assays were performed in RPMI/10% FCS.

- 30 Cytokines. Recombinant human interleukin-2 (rIL-2) (Sandoz) was used at a final concentration of 10 U/ml. Recombinant human interleukin-7 (rIL-7) (Genzyme) was used at a final concentration of 10 ng/ml.
- 35 Cultured Cell Lines. JY, a HLA A2.1 expressing human EBV-transformed B-cell line, was grown in RPMI/10% FCS. K562, a NK cell sensitive erythroblastoma line was grown in RPMI/10%

```
RCS. K562 was used to reduce background killing by MK and LAK release assays.
                                                                                                                                                                                                                                                                                                      The immunogenic peptides used in these studies were not if for un allowed and another for un allowed and another for un allowed another f
                                                                                                                                 Peptides. The immunogenic peptides used in these studies were notified in detail in detail in synthesized as described above using motified in detail in synthesized as described in detail in detai
                                                                                 res. Appl was used to release assays.
                                                                                                                                                   synthesized as described above using motifs for Him alle in detail in detail in for specific target antigens as as annications need with the specific target antigens and annications of the specific target and commonly assigned annications.
WO 95/04817
                                                                                                                                                                     for specific target antigens as described in detail in commonly assigned applications u.s.s.w.
                                                                                                                                                                                    copending and commonly assigned applications u.s.s.N. sequences are only assigned applications u.s.solved in 100 copending and commonly assigned applications were routinely dissolved in 100 copending and commonly assigned applications u.s.solved in 100 copending and commonly assigned applications u.s.sequences are of the copending and commonly assigned applications u.s.sequences are of the copending and commonly assigned applications u.s.sequences are of the copending and commonly assigned applications u.s.sequences are of the copending and commonly assigned applications u.s.sequences are of the copending and commonly assigned applications u.s.sequences are of the copending and commonly assigned applications u.s.sequences are of the copending and commonly assigned applications u.s.sequences are of the copending and commonly assigned applications u.s.sequences are of the copending and commonly assigned applications u.s.sequences are of the copending and commonly assigned applications u.s.sequences are of the copending and commonly assigned applications u.s.sequences are of the copending and commonly assigned applications u.s.sequences are of the copending and commonly assigned applications u.s.sequences are of the copending and commonly assigned applications u.s.sequences are of the copending applications 
                                                                                                                                                                                                         07|926,666 and U.S.S.N. 08|027,146 and their sequences are in 100% were routinely dissolved in 100% of peptides were routinely alicanoted at -20°C.

Shown in Table 1. aliquoted, and stored at -20°C.
                                                                                                                                                                                                                                                         Isolation of Peripheral Blood Mononuclear containing syringes in heparin (10 U/ml) containing syringes
                                                                                                                                                                                                                     snown in Table 1. Peptides were routinely dissolve aliquoted, and stored at 20 mg/ml, aliquoted, and stored at pms0 mg/ml, aliquoted, ali
                                                                                                                                                                                                                                                                        Tsolation of peripheral Blood Mononuclear Cells (PBMC) whole at 1600 relation of peripheral in heparin (10 U[ml) containing at 1600 relation of peripheral in heparin (10 U[ml) containing at 1600 relation of peripheral in heparin (10 U[ml) containing at 1600 relation of peripheral heparin (10 U[ml) containing at 1600 relation of peripheral heparin (10 U[ml) containing at 1600 relation of peripheral blood was collected in heparin (10 U[ml) containing at 1600 relation of peripheral heparin (10 U[ml) containing at 1600 relation of peripheral heparin (10 U[ml) containing at 1600 relation of peripheral heparin (10 U[ml) containing at 1600 relation of peripheral heparin (10 U[ml) containing at 1600 relation of peripheral heparin (10 U[ml) containing at 1600 relation of peripheral heparin (10 U[ml) containing at 1600 relation of peripheral heparin (10 U[ml) containing at 1600 relation of peripheral heparin (10 U[ml) containing at 1600 relation of peripheral heparin (10 U[ml) containing at 1600 relation of peripheral heparin (10 U[ml) containing at 1600 relation of peripheral heparin (10 U[ml) containing at 1600 relation of peripheral heparin (10 U[ml) containing at 1600 relation of peripheral heparin (10 U[ml] containing at 1600 relation of peripheral heparin (10 U[m] containing at 1600 relation of peripheral heparin (10 U[m] containing at 1600 relation of peripheral heparin (10 U[m] containing at 1600 relation of peripheral heparin (10 U[m] containing at 1600 relation of peripheral heparin (10 U[m] containing at 1600 relation of peripheral heparin (10 U[m] containing at 1600 relation of peripheral heparin (10 U[m] containing at 1600 relation of peripheral heparin (10 U[m] containing at 1600 relation of peripheral heparin (10 U[m] containing at 1600 relation of peripheral heparin (10 U[m] containing at 1600 relation of peripheral heparin (10 U[m] containing at 1600 relation of peripheral heparin (10 U[m] containing at 1600 relation of peripheral heparin (10 U[m] containing at 1600 relation of peripheral heparin (10 U[m
                                                                                                                                                                                                                                                                                             blood was collected in heparin (10 V/ml) containing at 1600 rpm tubes (Falcon) at removed and spun in 50cc conical rhe plasma layer was then removed and spun GS-6KR) 15 min.
                                                                                                                                                                                                                                                                                                                              (Beckman GS-6KR) 15 min.

The plasma layer was then removed with a 10 ml pipette with a mixed thorough the buffy coat was mixed the buffy coat was mixed thorough and 10 ml of the buffy mation.

The plasma layer was then removed the pipette with a 10 ml pipette 
                                                                     5
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         The buffy coat was mixed thoroughly
                                                                                                                                                                                                                                                                                                                                                                  using a circular motion.

The buffy coat was mixed thorough free RPWI 1640.

The buffy serum free RPWI 1640.

The buffy coat was mixed thorough free RPWI 1640.

The buffy coat was then lavered over 20 ml ricollers and diluted buffy coat was then lavered over 30 ml ricollers and diluted buffy coat was then lavered over 30 ml ricollers.
                                                                                                                                                                                                                                                                                                                                                                                    and diluted with an equal volume of serum free RPMI 1640.

and diluted with an equal then layered and centrifuced 400xq for the diluted buffy coat was conical tube and centrifuced diluted buffy in a socc conical tube
                                                                                                                                                                                                                                                                                                                                                                                                      diluted buffy coat was then layered over 20 ml Ficoll-Paque to tube and centrifuged 400xg me tube and the brake off me (Pharmacia) in a 50cc conical without the brake of (Pharmacia) at room temperature without the brake of the properature without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the pharmacial in a 50cc conical without the brake of the b
                                                                                                                                                                                                                                                                                                                                                                                                                     (Pharmacia) in a 50cc conical tube and centrifuged 400xg for the brake off. transformation at the brake using a transformation of temperature without the brake using a transformation of the prince was collected using a transformation of the prince without the brake of the prince without the brake of the prince without the brake of the prince without the prince without the prince of the prince without the prince of the prince of the prince without the prince of t
                                                                                                                                                                                                                                                                                                                           (Beckman GS-6KR) 15 min.
                                                                                                                                    10
                                                                                                                                                                                                                                                                                                                                                                                                                                       20 minutes at room temperature without the brake off. transfer times at room temperature was collected using a times times and washed three times and washed three times interface containing the per socc tube; and washed interfaces per socc tube; and washed three pipet (two interfaces per socc tube).
                                                                                                                                                                                                                                                                                                                                                                                                                                                   interface containing the PRMCs was collected using a transfer to times and washed three for 10 interfaces per 50cc tube) and 1300 rom for 10 pipet (two interfaces premi (1700. 1500. and 1300 rom firee pipet 50 ml serum free RPMI (1700. 1500. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. 1500. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. 1500. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. 1500. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 50 ml serum free RPMI (1700. and 1300 rom for 10 pipet 
                                                                                                                                                                                                                                                                                                                                                      using a circular motion.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                        pipet (two interfaces per 50cc tube) and 1300 rpm for 10 with 50 ml serum free RPMI (1700, 1500, and 1300 rpm free minutes.
                                                                                                                                                                                                                                 15
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          Freezing and Thawing to Cryovials were placed in Cryo 1°C freezing and Thawing to Cryovials were placed in Cryo 1°C freezing cells | ml of (Nalge).
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            cells/ml of 90% FCS to cryovials were placed (Fisher) and placed cryovials (Nalge) containing isopropanol (Fisher) containing contai
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              cryovials (Nalge) . Cryovials were placed in cryo loc freezing isopropanol (Fisher) and placed in cryo loc freezing isopropanol (maximum) to overnicht (maximum)
                                                                                                                                                                                                                                                                                                                        20
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                containers (Naige) containing isopropanol (Fiener) and post (maximum).

to overnight (maximum) to overnight (crvoviale overnight) to overnight (crvoviale overnight) to overnight (crvoviale overnight) to overnight (maximum) to ove
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 Freezing and Thawing PBMC.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             transferred to liquid nitrogen for in a 37°C water bath until transferred to continuous shaking cells were immediately thawed.

Were that crystal was nearly that the last crystal was nearly the last
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 Isopropanol was changed after every juses. cryovials term storage. hat liquid nitrogen for long 27°0 water hat transferred to more inverse charing in a 27°0 water hat transferred his continuous charing in a 27°0 water hat ware transferred his continuous charing in a 27°0 water hat he was a storage.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      minutes.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               at 10°C from 4 nours (minimum) to overnight overy 5 uses.

Teopropanol was 14 mil a mil manner of the minimum o
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    the last crystal was nearly thawed. Cells were immediately thawed. Cells were jumped and washed the last crystal was nearly thawed. Cells (Calbiochem) and washed the last crystal was nearly thawed. (Calbiochem) and washed diluted into serum by dead cells) (Calbiochem) and washed diluted into avoid clumning by dead cells)
                                                                                                                                                                                                                                                                                                                                                                                                            25
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         diluted into serum free RPNI medium (calbiochem) and washed (to avoid clumping by dead cells) (to avoid twice.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        were maked by containable thawed.

The last orystal was nearly the last orystal was a reserved to the last or the l
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                30
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 35
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    twice.
```

```
Preparation of CD4+ T cell ended to was performed using antibody-coated
                                                                                Preparation of CD4+ Trell depleted sessonder cell population.

Alasks: MicrocEllector T-150 flasks for the selection of CD4+ for 
                                                                              CD4+ lymphocyte depletion was performed using antibody-coated according to the
                                                                            cells (Applied Immune Sciences) were washed according to the page (CMP) (Calcium the
                                                                         cells (Applied manufacturer's immune Sciences) were washed according to swirling flasks for
                                                                 5
                                                                      magnesium free) instructions with 25 ml PBS CMF (calcium for 1 hour at room temberature on a
                                                                    sec followed by 1 mm EDTA (Sigma) by swirling flasks for 30 and flasks were washed 2
                                                                                                                                                                                                                 PCT/US94/08672
                                                                   flat surface.
                                                                additional times by shaking the flasks were washed of the binding surface. To each washe
                                                             maintaining times by shaking the flasks for 30 seconds and incubated for 20
                                                                                                   Buffer was aspirated and flasks were washed ?
                                                           Flask, 25 ml coverage of the binding surface.

Maintaining coverage of the binding surface.

Toom temperature on a flat surface.

Maria was lated for 20
                                                        in the flask until it was ready to receive the cells page.
                                                      in the flask until it was ready to receive the cells. PBMC
                                                    in the flask until it was ready to receive the culture medium containing 30 µg/ml DNAse and cells were
                                                  Washed thawed in culture medium containing 30 µg/ml DNAse and culture medium. Culture medium of 12 x 107 cells were
                                       15
                                               resuspended in 25 ml culture medium.
                                             aspirated in 25 ml culture medium.

dentily added to the Microcritector. Flasks containing was
                                          gently added from the flask and then the cell suspension was incubated for 1 hour at room temperature on a flat
                                        cells were incubated for 1 hour at room temperature on a flat was quently
                                      cells were incubated for 1 hour at room temperature on a flat resuspend the
                           50
                                    surface.

rocked from side end of the incubation, the flask was gen to resuspend the colls.

Nonadherent CD4+ T cell depleted cells
                                 rocked from side to side for 10 seconds to resuspend the washed twice with PRS CE
                               were harvested and then flasks were washed twice with PBS CMF
                             to collect the and then flasks were washed twice with PRG centrifucation and resuspe
                          to collect the depleted the nonadherent cells. In culture medium. Pelleted by centrifugation and resuspended
               25
                         in culture medium.
                   Standard Picoll-Damie Archocol PBMC Were isolated using the
                 standard Ficoll-Paque Protocol.
              Etandard Ficoli-Paque Protocol.

RPMI/5% HS containing 1 ua/m1 PHA (Well come) and 10 U/m1
    30
            RPMI/5% HS containing 1 were cultured at 2 x 10°/ml in blasts were maintained in culture and 10 U/ml
         TIL-2. PHA blasts were maintained in culture medium enlithing and lo
       containing 10 U/ml rIL-2 with feeding and splitting as needed.

PHA hlasts were used as APCs on day 6 of culture. Generation
     CONTAINING 10 U/MI FIL-2 WITH TEERING AND SPILLTING AND NORTH TO I PARISH WERE AND NORTH TO I PARISH WAS APPLICABLE.
  of empty class I molecules and peptide for culture.

Action method when inside parties only.
performed by the acid strip method when using PBMCs as APCs.
                                                                                                                               c_{e_{n_{e_{r_{\hat{a}}t_{i_{o_{n}}}}}}}
```

10

15

20

Acid Stripping/Peptide Loading of PBMC and PHA Blasts. PBMC were isolated using the Ficoll-Paque protocol. When using frozen cells, PBMC were washed twice before using. were prepared as previously described and washed twice before using. Once cells were prepared, they were washed once in cold sterile 0.9% NaCl (J.T. Baker) + 1% BSA. In a 50cc conical centrifuge tube, the cells were resuspended at 10⁷/ml in cold sterile citrate-phosphate buffer [0.13 M citric acid (J.T. Baker), 0.06 M sodium phosphate monobasic (Sigma) pH 3, 1% BSA, 3 μ g/ml β 2microglobulin (Scripps Labs)] and incubated for 2 minutes on ice. Immediately, 5 volumes of cold sterile neutralizing buffer #1 [0.15 M sodium phosphate monobasic pH 7.5, 1% BSA, 3 μ g/ml β 2microglobulin, 10 μ g/ml peptide] were added, and the cells were pelleted at 1500 rpm, 5 min at 4°C. Cells were resuspended in 1 volume cold sterile neutralizing buffer #2 [PBS CMF, 1% BSA, 30 µg/ml DNAse, 3 µg/ml β_2 microglobulin, 40 μ g/ml peptide and incubated for 4 hours at 20°C. Cells were diluted with culture medium to approximately 5 x $10^6/ml$ and irradiated with 6000 rads. were then centrifuged at 1500 rpm for 5 minutes at room temperature and resuspended in culture medium. The acid stripped/peptide loaded cells were used immediately in the CTL induction cultures (below).

25 Binding Assays Using Intact Cells and Radiolabelled Peptide. JY cells were either acid stripped (i.e. treated with citrate-phosphate buffer and neutralizing buffer #1 as described above) or incubated at a reduced temperature. JY control cells were left untreated in tissue culture media. After treatment both cell populations were washed twice with 30 serum free RPMI and loaded with 125I-radiolabelled 941.01 (HBc 18-27) peptide (standard chloramine T iodination). determine binding specificity, 2 x 10⁶ cells were resuspended in 200 μ l neutralizing buffer #2 (described'above) containing 125 1-941.01 (10⁵ cpms) +/- 100 μ g unlabelled 941.01. Cells 35 were incubated for 4 hours at 20°C and washed twice with serum free RPMI to remove free peptide. Cells were resuspended in 200 µl of serum free RPMI. In a microfuge tube the c ll

```
suspension was layered over an 800 µl rcs and pelleted by supernatants were against supernatants supernatants
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         Supernatants were aspirated and
                                                                                                 centrifugation for 5 seconds. Supernatants were aspirated supernatants were aspirated the radioactivity remaining counter. I minutes ner tune the radioactivity automatic damma counter.
                                                                                                                     the radioactivity remaining in the pellet was measured tube).
                                                                                                                                                 Binding of Radiolabeled peptides to Empty MHC Molecules.

Binding of Radiolabeled peptides loading using the column and the column and the service and the column and the service to Empty MHC Molecules.

Binding of Radiolabeled peptides to Empty MHC Molecules.

Binding of Radiolabeled peptides to Empty MHC Molecules.
                                                                                                                                                                  Binding of Radiolabeled peptides to Empty MHC Wolecules. To Empty MHC Wolecules to Empty MHC Wing the cold loading using the loading bentide loading bentide loading the efficiency acid stripping pentide loading determine the incubation or acid stripping the incubation of the temperature incubation of the loading determine incubation determine determine incubation determine determine incubation determine determi
                                                                                     centrifugation for 5 seconds.
WO 95/04817
                                                                                                                                                                                 determine the efficiency of peptide loading peptide loading the cold loading peptide loading peptide loading l
                                                                                                                                                                                                  temperature incubation or acid stripping peptide loading incubation or acid stripping peptide B cell line to remove the stripping peptide acid stripping peptide loading incubation or acid stripping peptide acid stripping peptide loading incubation or acid stripping peptide acid stripping peptide acid stripping peptide loading incubation or acid stripping peptide acid stripping acid strippin
                                                                                                                                                                                                                Protocol, TV cells (an HIA-A2.1 EBV-transformed B cell line)

Protocol, TV cells (an HIA-A2.1 EBV-transformed B cell line)

Protocol, TV cells (an HIA-A2.1 EBV-transformed B cell line)

Protocol, TV cells (an HIA-A2.1 EBV-transformed B cell line)

Protocol, TV cells (an HIA-A2.1 EBV-transformed B cell line)

Protocol, TV cells (an HIA-A2.1 EBV-transformed B cell line)

Protocol, TV cells (an HIA-A2.1 EBV-transformed B cell line)

Protocol, TV cells (an HIA-A2.1 EBV-transformed B cell line)

Protocol, TV cells (an HIA-A2.1 EBV-transformed B cell line)

Protocol, TV cells (an HIA-A2.1 EBV-transformed B cell line)

Protocol, TV cells (an HIA-A2.1 EBV-transformed B cell line)

Protocol, TV cells (an HIA-A2.1 EBV-transformed B cell line)

Protocol, TV cells (an HIA-A2.1 EBV-transformed B cell line)

Protocol, TV cells (an HIA-A2.1 EBV-transformed B cell line)
                                                                                                                                                                                                                                    were preincubated at 26°C overnight or acid-stripped to remove endogenous mic-associated peptides and last-radiolahelle the endogenous nentine was determined using a last-radiolahelle exponencus nentine was determined using a last-radiolahelle exponencus nentine was determined using a last-radiolahelle exponencus nentine was determined using a last remove the exponencus nentine was determined using the last remove the exponencus nentine was determined using the last remove the exponence of the last remove the endogenous nentine was determined using the last remove the endogenous nentine was determined using the last remove the endogenous nentine was determined using the last remove the endogenous nentine was determined using the last remove the endogenous nentine was determined using the last remove the endogenous nentine was determined using the last remove the endogenous nentine was determined using the last remove the endogenous nentine was determined using the last remove the endogenous nentine was determined using the last remove the endogenous nentine was determined using the last remove the endogenous nentine was determined using the last remove the endogenous nentine was determined using the last remove the endogenous nentine was determined using the last remove the endogenous nentine was determined to the endogenous ne
                                                                                                                                                                                                                                                        the endogenous MMC-associated peptides and the loading of the endogenous MMC-associated peptides and the loading of this result of this resul
                                                                                                                                                                                                                                                                    exogenous peptide was determined using a war-radiolabelled nent of this reaction of labelled nent the inhibition of labelled n
                                                                                                                                                                                                                                                                                     HIA-A2.1 binding peptide. The specificity of this reaction of labelled peptide inhibition of labelled Results the inhibition of Results was determined by measuring of the same segmence.
                                                                                                                                                                                                                                                                                                                    binding using a cold peptide of the same sequence. Results the tat acid-treatment of the binding using a cold peptide of that acid-treatment of the presented in Table 2 demonstrate (approximately 10-fold) the presented in Table 2 demonstrate (approximately 10-fold) the cells increased significantly (approximately 10-fold) the presented increased significantly (approximately 10-fold) the cells increased significantly (a
                                                                                                                                                                                                                                                                                                      was determined by measuring the inhibition of labell was determined by measuring the the same sequence.

Aemonetrate that acid-treatment in mahle 2 demonetrate that acid-treatment in make the same acid-treatment in the same acid-treat
                                                                                                                                                                                                                                                                                                                                     Presented in Table 2 demonstrate that acid-treatment of the Ty cells.

Presented in Table 2 demonstrate that acid-treatment of the Ty cells.
                                                                                                                                                                                                                                                                                                                                                                        amount of labelled peptide binding to the JY cells. Ompletely labelled peptide binding of labelled peptide demonstrating of the cold peptide. demonstrating furthermore, the addition of the blocked by the addition of the cold peptide.
                                                                                                                                                                                                                                                                                                                                                         cells increased significantly (approximately 10-fold to the JY cells.

amount of labelled peptide parallad nentide was and amount of labelled the hinding of labelled the hind
                                                                                                                                                                                                                                                                                                                                                                                      Furthermore, the binding of the cold peptide, demonstrating blocked by hinding (data not shown).
                                        , 21
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        Approximately 106 cells were used for each
                                                                                                                                                                                                                                                                                                                                                                                                                                                           FACS Analysis. Approximately 10° cells were washed twice to be tested.

To each sample. 100 ul PBS CMF + 0.1% BSA.

antibody antibody + 0.1% BSA.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          be rested. Cells were wasned twice with to .1% BSA TO each sample! Arcci or (0.12.1.
                                                                                                                                                                                                                                                                                                                                                                                                                Epecific binding (data not shown).
                                                                                                                                                                                                                         15
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          PBS CMF + 0.1% BSA. To each sample, 100 µl PBS CMF + 0.1

To each sample, 100 µl PBS CMF + 0.1

To each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each sample, 100 µl PBS CMF + 0.1

TO each samp
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           * Primary antibody at 2 µg/ml (BB1.21 Arcc) or (9.12.1)

* Primary antibody at 2 µg/ml (LB3.11 control was always INSERW-CNRS, were added.

* Primary antibody at 2 µg/ml (LB3.11 control was always primary antibody at 2 µg/ml (LB3.11 control was always always primary antibody at 2 µg/ml (LB3.11 control was always always primary antibody at 2 µg/ml (LB3.11 control was always always primary antibody at 2 µg/ml (LB3.11 control was always always primary antibody at 2 µg/ml (LB3.11 control was always always always always are added.)
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         INSERM-CNRS, Marseille) or (LE3.1, Children, S Hospital, Children, Children, S Hospital, Children, Children,
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             Pitteburgh) were added. A negative control was always and cells were included. Cells were CMF + 0.1% BSA. Cells were remember included.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            Cells Were resuspended
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             washed twice with PBS CMF + 0.1% BSA. and incubated 20 minutes on ice.

washed twice with PBS CMF + 0.1% BSA. and incubated 20 minutes on ice.

in 100 µl anti-mouse IgG and incubated 20 minutes on ice.
                                                                                                                                                                                                                                                                                                                                                                                                                                                  FACS Analysis.
                                                                                                                                                                                                                   ,41.77
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    PES CMF + 0.1% ESA.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         in PBS CMF + 0.1% BSA, and incubated 20 minutes on ice. cells in PBS CMF + 0.1% BSA, and incubated 20 minutes on ice. deals incubated 20 minutes on ice. deals incubated 20 minutes on ice. when it was incubated 20 minutes on ice. when it was incubated 20 minutes on ice. when it was incubated 20 minutes on ice. deals incubated 20 m
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              in 100 µl anti-mouse IgG FITC conjugate minutes on ice.

in 100 µl anti-mouse BSA, and incubated 20 minutes and resuspending PBS CMF + 0.1% BSA, and resuspendi
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               washed twice with pas care
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           Were washed twice with PBS CMK + 0.1% BSA, and resuspended when it was analysis.

When it was when it was analysis to the subsequent days the subsequent days. The subsequent days to the subsequent days analysis to the subsequent days.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            PBS for FACScan (Becton Dickinson) analysis. When it was the subsequent days, and the subsequent (Fisher) and necessary to postpone analysis paraformaldehyde (Fisher) necessary to postpone with PBS/1% paraformaldehyde (Fisher) and necessary to postpone with page 10 page
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             necessary to postpone analysis to the subsequent days, and recessary to postpone analysis to the subsequent (Fisher) and paraformal dehyde (Fisher) and cells were fixed within one week.
                                                                                                                                                                                                                                                                                                                                                                                         25
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 30
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    analyzed within one week.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               35
```

```
PHA-induced T-cell blasts were
                                                                 Measurements by FACS Analysis according to the methods were stained for acid stripped peptide resulting cells were stained above.
                                                                                                                                                                                                                                                                                                                           The resulting collaboration of 
                                                                                           described above. The resulting cells were stained for control and anti-HLA-A2 (BB7.2) and anti-HLA-A2 (BB7.2) antibodies. Control analysis using anti-HLA-A2 (BB7.2) antibodies.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       controls for
                                                                                                                       chain-specific (9.12.1) monoclonal antibodies. controls was this experiment at nH 3 (but treated with page buffer at nH 3 (but treated with page b
                                                          Measurements by FACS Analysis.
                                                                                                                                     this experiment included the same cell population which was the same cell population at pH 7.2);

this experiment included treated with pBS buffer (to strin the treated at pH 3 (but treated one treated at pH 3 (but trate-one of the treated at pH 3 (but citrate-one of the treated of treated of the treated 
                                                                                                            analyele using anci-HLA-AZ (BB1.2) and anci-HLA ancisons ancisons anci-HLA-AZ (BB1.2) and anci-HLA-AZ (BB1.2) anci-HLA
WO 95104817
                                                                                                                                                    not treated at pH 3 (but treated with pBS buffer (to strip the strip the at pH 3 (but treated with pBS buffer (to strip and cells treated with the absence of R.microalobulin and and cells treated in the absence of Maniero
                                                                                                                                                                  and cells treated with citrate-phosphate buffer (to strip the absence of $2 microglobulin and the absence in Figure 1. indicate that MHC) but neutralized in presented in presented periods.
                                                                                                                                                                             MRC) but neutralized in the absence of $2microglobulin and that absence of $2microglobulin that (nH3)

The absence of Figure 1, indicate (nH3)

The results presented in citrate-phosphate (nH3)

Peptide. The results with the citrate-phosphate (nH3)
                                                                                      described above.
                                                                                                                                                                                            Peptide. The results presented in riqure 1, indicate (RM3)

The results presented in citrate-phosphate (RM3)

treatment of these cells with (10-fold) the reactivity of treatment of these buffer significantly reduced (10-fold)
                                                                                                                                                                                                          treatment of these cells with the citrate-phosphate (pM3) the buffer significantly reduced class I antibodies alone buffer toward both anti-MIA class I
                                                                                                                                                                                                                                        cells toward both anti-HLA class I antibodies alone towards a and the alpha chain specific for class II MRC molecules (anti-HLA-A2 and the specific for class II monoclonal antibody specific
                                                                                                                                                                                                                          buffer significantly reduced (10-fold) the reactivity reduced (10-fold) the reduced (10-
                                                                                                                                                                                                                                                  (anti-HIA-A2 and the alpha chain specific), but not towar alpha chain specific mic molecules alpha chain specific for class II Mic molecules the alpha chain specific for class II Mic molecules alpha chain specific for class II Mic molecules importantly. neutralization of the monoclonal antibody importantly. neutralization of the monoclonal anti-HIA-DR).
                                                          5
                                                                                                                                                                                                                                                                  monoclonal antibody specific for class II MHC molecules
monoclonal antibody specific for class II MHC molecules
importantly, neutralization of hamicroglobulin a
monoclonal antibody specific for class II MHC molecules
in the presence of hamicroglobulin a
monoclonal antibody specific for class II MHC molecules
in the presence of hamicroglobulin a
monoclonal antibody specific for class II MHC molecules
in the presence of hamicroglobulin a
cantibody specific for class II MHC molecules
in the presence of hamicroglobulin a
cantibody specific for class II MHC molecules
in the presence of hamicroglobulin a
cantibody specific for class II MHC molecules
acid-stripped cells in the presence of hamicroglobulin a
acid-stripped cells in the presence of hamicroglobulin acid-stripped cells in the hamicroglobulin acid-stripped
                                                                                                                                                                                                                                                                              (anti-HLA-DR). Wost importantly, neutralization of the amount of hymicroglobulin and in the presence of a significant amount of a significant amount of a cid-stripped in preservation of a significant amount of a cid-stripped in preservation of a significant amount of a cid-stripped in preservation of a significant amount of a cid-stripped in preservation of a significant amount of a cid-stripped in preservation of a cid-stripped in cid-
                                                                                                                                                                                                                                                                                            acid-stripped cells in the presence of $2microglobulin and of with only a 2.5-fold with only a 2.5-fold peptide resulted antibody-reactive sites.
                                                                                                                                                                                                                                                                                                        peptide resulted in preservation of a significant amount of a significant amou
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             The acid-treated cells
                                                                                                                                                                                                                                                                                                                                    decrease in fluorescence intensity. The acid-treated cells we similar results we remained viable, as measured by trypan blue exclusion to we remained viable, packed analysis.
                                                                                                                   10
                                                                                                                                                                                                                                                                                                                                                              forward lateral FACS scatter analysis. Similar results were frozen)

forward lateral FACS scatter analysis. Similar results frozen)

forward lateral FACS scatter analysis. Similar results frozen)

forward lateral FACS scatter analysis. Similar results were

forward lateral FACS scatter analysis. Similar results frozen)

forward lateral FACS scatter analysis. Similar results frozen

forward lateral FACS scatter analysis frozen

forward l
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          Similar results were
                                                                                                                                                                                                                                                                                                                        class in fluorescence intensity.
                                                                                                                                                                                                                                                                                                                                                                          obtained using EBV-transformed B cell lines, fresh (or frozente bind to either HEA-A2.1 or page and other pertides (which bind to either HEA-A2.1 or page and other not shown).
                                                                                                                                                                                                                                                                                                                                                  remained viable; as measured analysis.

forward lateral payers are armed a real 14
                                                                                                                                                                                     15
                                                                                                                                                                                                                                                                                                                                                                                                                      Induction of Primary CTL Blasts as Stimulators.

Induction of PRMCs or PHA Blasts as Stimulators.
                                                                                                                                                                                                                                                                                                                                                                                                                                              Autologous PRNCs or PHA Blasts as Stimulators. Acid described with blasts are cells with stripping peptide loading incubation of stimulator cells with stripping peptide the 4 nour incubation of stimulator cells with above.
                                                                                                                                                                                                                                                                                                                                                                                                                                  Induction of primary CTL using Acid Stripped/Pertide I Acid

Induction of PRIMARY CTL Blasts as Stimulators.

Autologous PRIMCS or PHA Blasts and pun nicete and pun nicete are paur are paur and pun nicete are paur are paur and pun nicete are paur and pun nicete are paur ar
                                                                                                                                                                                                                                                                                                                                                                                                                                                            stripping/pertide loading of PRMC and PHA blasts are described with includation of stimulator cells with above. The responder cell normation was prepared the above.
                                                                                                                                                                                                                                                                                                                                                                                            HIA-All (data not shown).
                                                                                                                                                                                                                                                                                                                                                                                                                                                                          above. During the 4 nour incubation of stimulator center of the responder cell population was prepared:

Peptide, para panc that were deriated of content of the responder that were deriated of content of the responder that were deriated of content of the responder that were deriated of the responder that were derived the responder that were derived the respondence of stimulator center of the respondence of the respo
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      Peptide, the responder that were depleted of resugnended in Responders were PBMC that were replied were responder resugnended in Responders were above.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        Responders were PRMC that were depleted of CDA+ To cells were resuspended in Responder cells were the responder of the responder of the responder and 1 ml of the responder (described above).
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    (described above). Responder cells were resuspended in cell tissue the responder tissue at 1 ml of the responder tissue the responder cells were resuspended in cell tissue the responder cells were resuspended in cells were responder cells were responder tissue the responder tissue and 1 ml of the responder tissue the cells were responder tissue the responder tissue the responder tissue and 1 ml of the responder tissue tissue the responder tissue tissue the responder tissue tissue the responder tissue tissue
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  culture medium at 3 x 10° | ml and 1 ml of the responder tissue were medium at 3 x 10° | ml and 1 ml of a 24-well tissue of a 
                                                                                                                                                                                                                                                                                                                           25
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          culture plate incubator at 37°C, stimulator APCS were placed in the incubator once irradiated, stimulator placed in was ready.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        Placed in the incubator once irradiated, stimulator Apcs were population was ready.
                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                Suspension was alspensed and pickinson).
                                                                                                                                                                                                                                                                                                                                                                                            30
                                                                                                                                                                                                                                                                                                                                                                                                                                                                35
```

100 m

30

35

resuspended in culture medium containing 20 ng/ml rIL-7 at $10^6/\text{ml}$ for the PBMC, or at 3 x $10^5/\text{ml}$ for the PHA blasts, 1 ml of stimulator cell susp nsion was added per well to the plates containing the responders. On day 7 after induction, 100 μ l culture medium containing 200 ng/ml rIL-7 was added to each well (10 ng/ml rIL-7 final). On day 10 after induction, 100 μ1 of culture medium containing 200 U/ml rIL-2 was added to each well (10 U/ml rIL-2 final).

10 Antigen Restimulation of CTL. On day 12-14 after the induction, the primary CTL were restimulated with peptide using autologous, adherent APCs. Autologous PBMC were thawedand washed as described above. Cells were irradiated at 6000 rads. Cells were pelleted and resuspended in culture medium 15 an at $4 \times 10^6/\text{ml}$ and 1 ml of cell suspension was added to each well of a 24-well tissue culture plate, and incubated for 2 hours at 37°C, 5% CO2. Nonadherent cells were removed by washing each well three times with serum free RPMI. After this step, a 0.5 ml culture medium containing 3 μ g/ml β_2 microglobulin and 20 μ g/ml total peptide was added to each 20 well. APC were incubated for 2 hrs at 37°C, under 5% CO, with the peptide and β_2 microglobulin. Wells were aspirated and 1 ml of responder cells at 1.5 x $10^6/ml$ in culture medium was added to each well. After 2 days, 1 ml of culture medium containing 20 U/ml rIL-2 was added to each well. Cultures 25 were supplemented with 10 U/ml rIl-2 (final) every three days thereafter.

> Cytotoxicity Chromium Release Assay. Seven days following restimulation of primary induction, the cytotoxic activity of the cultures was assessed.

Effector Cell Preparation: The responders were centrifuged and resuspended at 107/ml in RPMI/10% FCS. Three-fold serial dilutions of effectors were performed to yield effector to target ratios of 100:1, 33:1, 11:1, and 3:1. Effector cells were aliquoted at 100 μ l/well on 96 well U-bottom d cluster plates (Costar), in duplicate.

35

- b. Target Cell Preparation: Approximately 16-20 hours prior to the assay, target cells were resuspended at 3 x $10^5/\text{ml}$ in RPMI/10% FCS in the pres nce or absence of 3 $\mu\text{g/ml}$ β_2 microglobulin and 10 $\mu\text{g/ml}$ total peptide. After preincubation, target cells were centrifuged and pellets were resuspended in 200 μl (300 μCi) sodium (^{51}Cr) chromate (NEN). Cells were incubated at 37°C for 1 hour with agitation. Labelled target cells were washed 3 times with RPMI/10% FCS.
- Target cell Setting-Up the Assays: concentration was adjusted to $10^5/\text{ml}$ in RPMI/10% FCS and 100 10 μ l aliquots were added to each well containing responders. K562 cells (cold targets, to block NK, and LAK activity) were washed and resuspended in RPMI/10% FCS at 107/ml. Aliquots of 20 μ l were added per well, yielding a 20:1 cold K562 target to labelled target ratio. For the determination of the 15 spontaneous ⁵¹Cr release, 100 μl/well of RPMI/10% FCS were added to 100 μ l/well of labelled target cells, and 20 μ l/well of K562. For maximum 51 Cr release, 100 μ l 1% Triton X-100 (Sigma) in PBS CMF, was added to the 100 μ l/well labelled target cells, and 20 μ l/well K562. Plates were centrifuged 20 for 2 minutes at 1200 rpm to accelerate cell conjugate formation. Assays were incubated for 5 hours at 37°C, 5% CO2. Assays were harvested by centrifuging plates for 5 minutes at 1200 rpm and collecting 100 μ l/well of supernatant. Standard gamma counting techniques were used to determine percent 25 specific lysis (Micromedic automatic gamma counter, 0.5 minutes per tube). Percent specific lysis was determined by the following formula: cpm experimental release -cpm spontaneous release/cpm maximum release-cpm spontaneous 30 release x 100.

In Vitro Induction of Primary Antigen-Specific CTL Using Acid Stripped/Peptide Loaded APCS. Additional critical parameters for the induction of primary CTL are: 1) enrichment of CD8+T-cells in the responder cell population (by depletion of CD4+T-cells), 2) addition of rIL-7 to the CTL induction cultures from day 0, and 3) restimulation of the cultures with antigen on day 12-14 using autologous adherent cells pulsed with

peptide. Results presented in Figures 2, 3 and 5 correspond to experiments performed using PBMC as APC. The results presented in Figure 4 present results obtained using PHA-induced T-cell blasts as APC. Figure 7 shows a comparison of the acid strip loading technique (Figure 7a) to the cold temperature incubation technique (Figure 7b).

Example 3

Screening peptides to identify CTL epitopes.

10

, **15**

.

43.微· 游· 5

In order to identify CTL epitopes, CTL were stimulated by SAC-I activated PBMCs as APC. Cold temperature enhanced expression of empty MHC enabling loading of antigenic peptide to generate SAC-I activated PBMC APC. This method presents an alternative protocol to the methods described above for the generation of the APC which are used to stimulate CTL. This example also presents an alternative protocol for the stimulation of CTL by the APC.

- Complete Culture Medium. The tissue culture medium used in this study consisted of RPMI 1640 with Hepes and L-glutamine (Gibco) (Biowhittaker) supplemented with 2 mM L-glutamine (Irvine Scientific), 0.5mM sodium pyruvate (Gibco), 100 U/100 ug/ml penicillin/streptomycin (Irvine), and 5%
- heat-inactivated Human Serum Type AB (RPMI/5% HS; Gemini Bioproducts). Culture media used in the growth of EBV-transformed lines contained 10% heat-inactivated fetal calf serum (RPMI/10% FCS, Irvine) instead of human serum.
 - 30 Cytokines. Recombinant human Interleukin-2 (rIL-2) and Interleukin-4 (rIL-4) were obtained from Sandoz and used at a final concentration of 10 U/ml and 10 ng/ml, respectively. Human interferon-γ (IFN-γ) and recombinant human Interleukin-7 (rlL-7) were obtained from Genzyme and used at 20 U/ml and 10 ng/ml, respectively.
 - Peptid s. Peptides w r synthesized as described above and ar described in Tabl 1. Peptides were routin ly dissolv d

in 100% DMSO at 20 mg/ml, aliquoted, and stored at -70°C until used.

Cell Lines. JY, Steinlein, EHM, BVR, and KT3 are homozygous 5 human EBV-transformed B cell lines expressing HLA A2 1, A1, A3, A_{11} , and A_{24} , respectively. They are grown in RPMI/10% FCS and used as targets in the CTL assays. K562, an NK cell . sensitive, erythroblastoma line grown in RPMI/10% FCS, was used for reduction of background killing in the CTL assays. Melanoma HLA A1+ cell lines either expressing the MAGE antigen, mel 397 and mel 938 or those not expressing the MAGE antigen, mel 888, were also grown in RPMI/10% FCS.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs), 15 Whole blood was collected into heparin containing syringes and spun in 50cc tubes at 1600 RPM (Beckman GS-6KR) for 15 minutes. The plasma layer was then removed and 10 ml of buffy coat was collected with a pipette using a circular motion. The buffy coat was mixed well and diluted with an equal volume 20 of RPMI. The buffy coat (30 ml) was then layered on 20 ml of Ficoll-Paque (Pharmacia) and centrifuged at 1850 RPM (400xg) for 20 minutes, 25°C, with the brake off. The interface between the Ficoll and the plasma containing the PBMCs was recovered with a transfer pipet (two interfaces per 50 ml 25 tube) and washed three times with 50 ml of RPMI (1700, 1500, and 1300 RPM for 10 minutes). Cells were resuspended in 10-20 ml of culture medium, counted, and adjusted to the appropriate concentration.

Freezing PBMCs. 30 million cells/tube (90% FCS/10% DMSO; 30 Sigma) were inserted into a Nalgene Cryo 1°C Freezing Container containing isopropanol (Fisher) and placed at -70°C from 4 hrs (minimum) to overnight (maximum). The isopropanol was changed every five times. Tubes were transferred to liquid nitrogen for long term storage. To thaw, PBMCs were 35 continuously shaken in a 37°C water bath until the last crystal was almost thawed (tubes were not allowed to sit in the water bath or at room temperature for any period of time).

10

15

25

30

35

Cells were diluted into serum-free RPMI containing 30 μ g/ml DNase to prevent clumping by dead cell DNA and washed twice.

Induction of Primary CTL Using SAC-I Activated PBMCs as APCs

a. <u>Preparation of SAC-I activated PBMCs as APCs</u>: PBMCs were purified using the standard Ficoll-Paque protocol and resuspended at 1 x 10⁶/ml in RPMI/5% FCS containing 0.005% Pansorbin cells (SAC-I cells expressing Protein A; Calbiochem), 20 µg/ml Immunobeads (Rabbit anti-Human IgM; Biorad), and 20 ng/ml of human rIL-4. Two ml of cells per well were plated in a 24-well plate (Falcon, Becton Dickinson) and cultured at 37°C. After 3 days, the medium was removed and the cells were washed three times followed by addition of RPMI/10% HS. The cells were used after culturing for an additional 2 days in RPMI/10% HS.

b. Expression of empty Class I molecules on the surface of APCs and peptide loading of APCs.

1. Cold temperature incubation:

a. Expression of empty MHC in APCs: The APCs were adjusted to a concentration of 2 x $10^6/\text{ml}$ in complete culture medium containing 10 ng/ml rIL-4, 20 U/ml human IFN- γ , and 3 μ g/ml β 2-microglobulin (β 2m; Scripps Lab). The cells were then incubated overnight at 26°C in the presence of 5% CO₂. It should be noted that these cells only express a fraction of Class I molecules in the empty state (~10%).

b. Peptide loading of APC stimulator cells: Empty Class I expressing APCs were washed 1-2 times with serum free RPMI (+ L-glutamine and Hepes) and resuspended at 1 X 10^7 in serum-free RPMI containing 50 μ g/ml total of the peptide pool (i.e., $16.7~\mu$ g/ml of each peptide in a pool of three; 25 μ g/ml of each peptide in a pool of two; 50 μ g/ml of individual peptide), 30 μ g/ml DNAse, and 3 μ g/ml β_2 m. Following a 4 hour incubation at 20°C, the cells were irradiated at 6100 rads (5 x 10^6 / ml; 25 million cells/tube), washed and adjusted to the appropriate concentration for addition to the induction culture (see b low).

2. Acid stripping: This was used as an

10

15

20

25

30

35

alternative method for generating empty MHC on the surface of The SAC-I activated PBMCs were washed once in cold 0.9% sodium chloride (J.T. Baker) containing 1% BSA. cells were resuspended at 10⁷/ml in cold citrate-phosphate buffer (0.13M citric acid [J.T. Baker], 0.06M sodium phosphate monobasic [Sigma], pH3) containing 1% BSA and 3 μ g/ml β_2 m and incubated on ice. After 2 minutes, 5 volumes of cold 0.15M sodium phosphate buffer, pH7.5, containing 1% BSA, 3 μ g/ml β_2 m, and 10 μ g/ml peptide [neutralizing buffer #1] was added and the cells centrifuged at 1500 RPM for 5 minutes at 4°C. The cells were resuspended in 1 ml of cold PBS containing 1% BSA, 30 μ g/ml DNase, 3 μ g/ml β_2 microglobulin, and 50 μ g/ml peptide [neutralizing buffer #2] and incubated for 4 hours at 20°C. As above, subsequent to the four hour incubation at 20°C, the cells were irradiated at 6100 rads (5 \times 10⁶/ ml; 25 million cells/tube), washed, then adjusted to the appropriate concentration for addition to the induction culture (see below) .

Preparation of the CD4+ depleted PBMC responder cell population (depletion of lymphocyte subpopulations using AIS flasks). AIS MicroCellector T-150 flasks (specific for the depletion of CD4+ T cells; Menlo Park, CA) were primed by adding 25 ml of PBS/1 mM EDTA, swirling for 30 seconds so that all surfaces were moistened, and then incubating with the binding surface down at room temperature for 1 hour. Following this incubation, flasks were shaken vigorously for 30 seconds, washed 1 time with PBS/EDTA, 2 additional times with PBS and then incubated with 25 ml of culture medium for 15 minutes. PBMCs were thawed in serum-free RPMI (+ L-glutamine + Hepes) containing 30 μ g/ml DNAse, washed once, and incubated for 15 minutes in culture medium. Following aspiration of culture medium from the flasks, up to 180 million PBMCs were added in 25 ml of culture medium containing 30 μ g/ml DNAse. After 1 hour at room temperature, the flasks were rocked gently for 10 seconds to resuspend the nonadherent cells. The nonadher nt c ll suspension containing the CD8+ T cells was c llected and the

15

20

25

30

35

flasks were washed 2 times with PBS. The CD4+ T cell d pleted PBMCs were centrifuged and counted for addition to the induction culture. The CD4+ and CD8+ phenotype of the CD4+ depleted cell population was determined by FACS analysis (see below). In general, this technique resulted in a two-fold enrichment for CD8+ T cells with an average of approximately 40-50% CD8+ T cells and 15-20% remaining CD4+ T cells following depletion of CD4+ T cells. Depletion of CD4+ T cells can also be accomplished by using antibody and complement methods or antibody coated magnetic beads (Dynabeads). Depletion of CD4+ T cells enriched the CTLp and removed cells which competed for cell nutrients.

Induction of primary CTL. During the 4 hour peptide loading of the stimulator APCs, CD4+ depleted PBMC to be used as the responder population were prepared utilizing AIS flasks for selection of CD8+ T cells through the depletion of CD4+ T cells (above). The responder cells were plated at 3 \times 10⁶/ml in a 1 ml volume (24 well plate) and placed at 37°C until the peptide loaded stimulator APCs were prepared. The irradiated, peptide loaded APCs were washed 1 time in serum-free RPMI (+ L-glutamine and Hepes), adjusted to the appropriate concentration in complete medium, and plated into a 24 well plate at 1 ml/plate: For PBMC and SAC-I activated PBMCs as APCs 1 x 106 stimulator cells (1 ml volume) were plated into the wells containing the responder cells; For PHA blasts as APCs, 1 ml of 3 x $10^5/ml$ stimulator cells were plated in each well. A final concentration of 10 ng/ml of rIL-7 (2 ml total volume) was added. On day 7 an additional 10 μ g/ml rIL-7 was added to the culture and 10 U/ml rIL-2 was added every 3 days thereafter. On day 12, the cultures were restimulated with peptide pulsed adherent cells and tested for cytolytic activity 7 days later (below).

Protocol for Restimulation of Primary CTL Using Autologous Adherent APC. Autologous PBMCs were thawed into serum-free RPMI (+ L-glutamine and Hepes) containing $30\mu g/ml$ DNAse, washed 2 times, and adjust d to 5 x 10^6 /ml in culture medium containing DNAse. PBMCs (25 million cells/tube in 5 ml) were

10

15

20

25.

30

35

irradiated at 6100R. After 1 wash, the PBMCs were resuspended in culture medium and adjusted to 4 x $10^6/\text{ml}$ and 1 ml of irradiated PBMCs was added per well of a 24-well plate. The PBMC were incubated for 2 hours at 37°C, washed 3 times to remove nonadherent cells, and cultured in medium containing 20 $\mu\text{g/ml}$ total peptide and 3 $\mu\text{g/ml}$ $\beta_2\text{microglobulin}$ added in a 0.5 ml volume and again incubated for 2 hours at 37°C. The peptide was aspirated and 1.5 x 10^6 responder cells resuspended in culture medium were added in a 1 ml volume. After 2 days, 1 ml of culture medium containing 20 U/ml rIL-2 was added.

FACS Analysis. One million cells/tube were centrifuged, resuspended in 100 μ l/tube PBS/0.1% BSA/0.02% sodium azide (Sigma) plus 10 μ l/tube directly conjugated antibody (Becton Dickinson), and incubated on ice 15-20 minutes. Cells were then washed 2 times with PBS/0.1% BSA/0.02% sodium azide and resuspended in PBS to analyze on FACScan (Becton Dickinson). When it was not possible to analyze samples within 1-2 days, cells were fixed with PBS containing 1% paraformaldehyde (Fisher) and analyzed within one week.

Cytotoxicity Assay

- a. <u>Target cell preparation</u>. Approximately 16-20 hours prior to the CTL assay, target cells (Class I matched EBV-transformed lines) were washed once and resuspended in a 10 ml volume at 3 x $10^5/\text{ml}$ in RPMI/5% FCS in the presence or absence of 10 $\mu\text{g/ml}$ total peptide.
- b. Labeling of target cells: Target cells were centrifuged and resuspended in 200 μ l/tube sodium ⁵¹Cr chromate (NEN), then incubated at 37°C for 1 hour on a shaker. Targets were washed 3 times (10 ml/wash) with RPMI/10% FCS and resuspended in 10 ml (to determine the efficiency of labelling, 50 μ l/target was counted on the Micromedic automatic gamma counter).
- c. <u>CTL assay</u>. Target cells were adjusted to $2 \times 10^5/ml$ and $50 \ \mu l$ of the cell culture was added to each well of a U-bottomed 96-well plate (Costar Corp.) for a final concentration of 1 \times $10^4/well$. K562 cells were washed once,

5

10

15

20

25

30

35

· y

33

resuspended at 4 x $10^6/ml$, and 50 μ l/well was added for a final concentration of 2 x 105/well (ratio of cold K562 to target was 20:1). Responder cells were washed once, resuspended at 9 x 10⁶/ml, and three fold serial dilutions were performed for effector to target ratios of 90:1, 30:1, 10:1, and 3:1. Responder cells were added in a volume of 100 μl in duplicate wells. For spontaneous release, 50 μl/well of labelled target cells, 50 μ l/well K562, and 100 μ l/well of medium was added. For maximum release, 50 \(\mu \) / well target, 50 μ l/well K562, and 100 μ l/well of 0.1% Triton-X100 (Sigma) was added. Plates were centrifuged for 5 minutes at 1200 RPM. Following a 5 hour incubation at 37°C, plates were centrifuged again for 5 minutes at 1200 RPM, and 100 μ l/well of supernatant was collected. Standard gamma counting techniques (Micromedic automatic gamma counter; 0.5 minutes/tube) were used to determine the percent specific lysis according to the formula: % specific lysis = cpm experimental release - cpm spontaneous release/cpm maximum release - cpm spontaneous release X 100. A cytotoxicity assay (CTL assay) was considered positive if the lysis by CTL of targets sensitized with a specific peptide at the two highest effector to target (E:T) ratios was 15% greater than lysis of control targets (i.e. target cells without peptide). A cytotoxicity assay (CTL assay) was considered borderline if the lysis by CTL of targets sensitized with a specific peptide at the two highest effector to target (E:T) ratios was 6% greater than lysis of control targets (i.e. target cells without peptide).

d. Results. Of the peptides that bind to the indicated alleles, 12 of the 60 MAGE peptides, 13 of the 53 HIV peptides, 3 of the 25 HCV peptides, and 7 of the 28 HBV peptides tested to date induced primary CTL in vitro. Representative graphs illustrating CTL responses to various immunogenic peptides are shown for MAGE (Figure 8), HIV (Figure 9), HCV (Figure 10), and HBV (Figure 11). The CTL induction data is summarized in Table 3 which lists the immunogenic peptides which bind to the appropriate MHC and induce primary CTL in vitro. Indicated is the peptide's sequence, corresponding antigen and HLA allele to which it

10

binds. Results shown in Figure 6 illustrate lysis of peptide sensitized targets and endogenous targets following stimulation with SACI activated PBMCs loaded with the immunogenic peptide MAGE-3 1044.07 which had been loaded using cold temperature incubation.

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

Table 1

927.32 HBVP 61-69 GLYSTVPV 938.01 MAGE 1 161-169 EADPTGHSY 939.03 PSA 49-57 VLVHPQWVL 941.01 HBVC 18-27 analog FLPSDYFPSV 1044.04 PAP 135-143 ILLWDPIPV 1044.05 PSA 166-175 KLQCVDLVHI 1044.06 PSA 118-128 MLLRLSEPAE 1044.07 MAGE 3 161-169 EVDPIGHLY 1072.13 MAGE 1 96-104 SLFRAVITK 1072.18 MAGE 1 66-74 TTINFTRQR 1072.20 MAGE 1 219-227 SVMEVYDGR 1072.39 MAGE 1 238-247 LLTQDLVQER 1072.15 MAGE 1 95-104 ESLFRAVITE	PBMCs and PHA Blasts		
## HBVC 18-27 FLPSDFFPSV ## P27.32 HBVP 61-69 GLYSSTVPV ## P38.01 MAGE 1 161-169 EADPTGHSY ## P39.03 PSA 49-57 VLVHPQWVL ## P41.01 HBVC 18-27 analog FLPSDYFPSV ## P41.01 PAP 135-143 ILLWDPIPV ## P41.05 PSA 166-175 KLQCVDLVHI ## P5A 118-128 MLLRLSEPAE ## P5A 118-128 MLLRLSEPAE ## P5A 118-128 MAGE 3 161-169 EVDPIGHLY ## P5A 13 P6-104 SLFRAVITK ## P6-104 SLFRAVITK ## P6-104 TTINFTRQR ## P6-105 PSA 1072.13 MAGE 1 219-227 SVMEVYDGR ## P6-105 PSA 1072.20 MAGE 1 219-227 SVMEVYDGR ## P6-105 PSA 1072.20 MAGE 1 238-247 LLTQDLVQER ## P6-105 PSA 1072.22 MAGE 1 238-247 LLTQDLVQER ## P6-105 PSA 1072.23 PSA 1072.23 PSA 1072.23 PSA 1072.23 PSA 1072.25 PSA 107	eptide ID #	Antigen	Sequence
927.32 HBVP 61-69 GLYSTVPV 938.01 MAGE 1 161-169 EADPTGHSY 939.03 PSA 49-57 VLVHPQWVL 941.01 HBVC 18-27 analog FLPSDYFPSV 1044.04 PAP 135-143 ILLWDPIPV 1044.05 PSA 166-175 KLQCVDLVHI 1044.06 PSA 118-128 MLLRLSEPAE 1044.07 MAGE 3 161-169 EVDPIGHLY 1044.01 MAGE 3 8-17 ASSLPTTMNY 1072.13 MAGE 1 96-104 SLFRAVITK 1072.18 MAGE 1 66-74 TTINFTRQR 1072.20 MAGE 1 219-227 SVMEVYDGR 1072.39 MAGE 1 238-247 LLTQDLVQER 1072.22 MAGE 1 238-247 LLTQDLVQER	777.03	HBVs 20-28	FLLTRILTI
938.01 MAGE 1 161-169 EADPTGHSY 939.03 PSA 49-57 VLVHPQWVL 941.01 HBVC 18-27 analog FLPSDYFPSV 1044.04 PAP 135-143 ILLWDPIPV 1044.05 PSA 166-175 KLQCVDLVHI 1044.06 PSA 118-128 MLLRLSEPAE 1044.07 MAGE 3 161-169 EVDPIGHLY 1044.01 MAGE 3 8-17 ASSLPTTMNY 1072.13 MAGE 1 96-104 SLFRAVITK 1072.18 MAGE 1 66-74 TTINFTRQR 1072.20 MAGE 1 219-227 SVMEVYDGR 1072.39 MAGE 1N 270-279 RALAETSYVE 1072.22 MAGE 1 238-247 LLTQDLVQEE 1072.15 MAGE 1 95-104 ESLFRAVITE	24.07	HBVc 18-27	FLPSDFFPSV
PSA 49-57 VLVHPQWVL 941.01 HBVC 18-27 analog FLPSDYFPSV 1044.04 PAP 135-143 ILLWDPIPV 1044.05 PSA 166-175 KLQCVDLVHI 1044.06 PSA 118-128 MLLRLSEPAE 1044.07 MAGE 3 161-169 EVDPIGHLY 1044.01 MAGE 3 8-17 ASSLPTTMNY 1072.13 MAGE 1 96-104 SLFRAVITK 1072.18 MAGE 1 66-74 TTINFTRQR 1072.20 MAGE 1 219-227 SVMEVYDGR 1072.39 MAGE 1N 270-279 RALAETSYVE 1072.22 MAGE 1 238-247 LLTQDLVQEE 1072.15 MAGE 1 95-104 ESLFRAVITE	927.32	HBVp 61-69	GLYSSTVPV
941.01 HBVC 18-27 analog FLPSDYFPSV 1044.04 PAP 135-143 ILLWDPIPV 1044.05 PSA 166-175 KLQCVDLVHI 1044.06 PSA 118-128 MLLRLSEPAE 1044.07 MAGE 3 161-169 EVDPIGHLY 1044.01 MAGE 3 8-17 ASSLPTTMNY 1072.13 MAGE 1 96-104 SLFRAVITK 1072.18 MAGE 1 66-74 TTINFTRQR 1072.20 MAGE 1 219-227 SVMEVYDGR 1072.39 MAGE 1 238-247 LLTQDLVQER 1072.22 MAGE 1 95-104 ESLFRAVITE	938.01	MAGE 1 161-169	EADPTGHSY
1044.04 PAP 135-143 ILLWDPIPV 1044.05 PSA 166-175 KLQCVDLVHI 1044.06 PSA 118-128 MLLRLSEPAE 1044.07 MAGE 3 161-169 EVDPIGHLY 1044.01 MAGE 3 8-17 ASSLPTTMNY 1072.13 MAGE 1 96-104 SLFRAVITK 1072.18 MAGE 1 66-74 TTINFTRQR 1072.20 MAGE 1 219-227 SVMEVYDGR 1072.39 MAGE 1N 270-279 RALAETSYVK 1072.22 MAGE 1 238-247 LLTQDLVQEK 1072.15 MAGE 1 95-104 ESLFRAVITE	939.03	PSA 49-57	VLVHPQWVL
PSA 166-175 KLQCVDLVHI 1044.06 PSA 118-128 MLLRLSEPAE 1044.07 MAGE 3 161-169 EVDPIGHLY 1044.01 MAGE 3 8-17 ASSLPTTMNY 1072.13 MAGE 1 96-104 SLFRAVITK 1072.18 MAGE 1 66-74 TTINFTRQR 1072.20 MAGE 1 219-227 SVMEVYDGR 1072.39 MAGE 1N 270-279 RALAETSYVK 1072.22 MAGE 1 238-247 LLTQDLVQER 1072.15 MAGE 1 95-104 ESLFRAVITE	941.01	HBVc 18-27 analog	FLPSDYFPSV
PSA 166-175 RIQCVDLVHI 1044.06 PSA 118-128 MLLRLSEPAE 1044.07 MAGE 3 161-169 EVDPIGHLY 1044.01 MAGE 3 8-17 ASSLPTTMNY 1072.13 MAGE 1 96-104 SLFRAVITK 1072.18 MAGE 1 66-74 TTINFTRQR 1072.20 MAGE 1 219-227 SVMEVYDGR 1072.39 MAGE 1 270-279 RALAETSYVE 1072.22 MAGE 1 238-247 LLTQDLVQEE 1072.15 MAGE 1 95-104 ESLFRAVITE	1044.04	PAP 135-143	ILLWDPIPV.
1044.07 MAGE 3 161-169 EVDPIGHLY 1044.01 MAGE 3 8-17 ASSLPTTMNY 1072.13 MAGE 1 96-104 SLFRAVITK 1072.18 MAGE 1 66-74 TTINFTRQR 1072.20 MAGE 1 219-227 SVMEVYDGR 1072.39 MAGE 1N 270-279 RALAETSYVK 1072.22 MAGE 1 238-247 LLTQDLVQER 1072.15 MAGE 1 95-104 ESLFRAVITE	1044.05	PSA 166-175	KLQCVDLVHI
1044.01 MAGE 3 8-17 ASSLPTTMNY 1072.13 MAGE 1 96-104 SLFRAVITK 1072.18 MAGE 1 66-74 TTINFTRQR 1072.20 MAGE 1 219-227 SVMEVYDGR 1072.39 MAGE 1N 270-279 RALAETSYVK 1072.22 MAGE 1 238-247 LLTQDLVQEK 1072.15 MAGE 1 95-104 ESLFRAVITE	1044.06	PSA 118-128	MLLRLSEPAEL
1072.13 MAGE 1 96-104 SLFRAVITK 1072.18 MAGE 1 66-74 TTINFTRQR 1072.20 MAGE 1 219-227 SVMEVYDGR 1072.39 MAGE 1N 270-279 RALAETSYVE 1072.22 MAGE 1 238-247 LLTQDLVQER 1072.15 MAGE 1 95-104 ESLFRAVITE	1044.07	MAGE 3 161-169	EVDPIGHLY
1072.18 MAGE 1 66-74 TTINFTRQR 1072.20 MAGE 1 219-227 SVMEVYDGR 1072.39 MAGE 1N 270-279 RALAETSYVE 1072.22 MAGE 1 238-247 LLTQDLVQER 1072.15 MAGE 1 95-104 ESLFRAVITE	1044.01	MAGE 3 8-17	ASSLPTTMNY
1072.20 MAGE 1 219-227 SVMEVYDGR 1072.39 MAGE 1N 270-279 RALAETSYVK 1072.22 MAGE 1 238-247 LLTQDLVQEK 1072.15 MAGE 1 95-104 ESLFRAVITE	1072.13	MAGE 1 96-104	SLFRAVITK
1072.39 MAGE 1N 270-279 RALAETSYVE 1072.22 MAGE 1 238-247 LLTQDLVQEE 1072.15 MAGE 1 95-104 ESLFRAVITE	1072.18	MAGE 1 66-74	TTINFTRQR
1072.22 MAGE 1 238-247 LLTQDLVQER 1072.15 MAGE 1 95-104 ESLFRAVITE	1072.20	MAGE 1 219-227	SVMEVYDGR
1072.15 MAGE 1 95-104 ESLFRAVITY	1072.39	MAGE 1N 270-279	RALAETSYVK
10/2.15	1072.22	MAGE 1 238-247	LLTQDLVQEK
1069.42 HIV pol 1225-1235 KVYLAWVPAH	1072.15	MAGE 1 95-104	ESLFRAVITK
	1069.42	HIV pol 1225-1235	KVYLAWVPAHK
1069.43 HIV env 2185-2194 TVYYGVPVWI	1069.43	HIV env 2185-2194	TVYYGVPVWK

1069.47	HIV env 2184-2194	VTVYYGVPVWK
1069.48	HIV pol 1434-1443	AVFIHNFKRK
1073.10	HCV LORF 1858-1867	GVAGALVAFK
1073.11	HCV CORE 43-51	RLGVRATRK
1073.16	HCV LORF 1227-1236	. HLHAPTGSGK
1069.15	HBV pol 724-733	ŤLWKAGILYK

5

Table 2

Acid Stripping, Peptide Loading of JY Cells with Radiolabelled 941.01				
Cell Population	125 _I -Labeled Peptide +/- Cold Peptide	CPMS +/- std. dev.		
JY acid stripped	- cold peptide	3553 ± 157	n = 3	
JY acid stripped	+ cold peptide	13	n = 1	
JY_control	-cold peptide	370 ± 37	n = .3	
JY control	+ cold peptide	50	n = 1	

5

Table 3

Sequence	Antigen	Motif	Id
EVDPIGHLY	MAGE3	A01	1044.07
ASSLPTTMNY	MAGE3	A01	1044.01
EADPTGHSY	MAGE1	A01	958.01
SSLPTTMNY*	MAGE3	A01	1072.02*
GSVVGNWQY *	MAGE3	A01	1072.03*
ALAETSYVK*	MAGE1N	A03	1072.38*
SLFRAVITK	MAGEI	All	1072.13
RALAETSYVK	MAGE1N	A11	1072.39
ESLFRAVITK	MAGE1	A11	1072.15
KVYLAWVPAHK	HIV	A3/11*	1069.42*
TVYYGVPVWK	HIV	A03	1069.43
Klagrwpvk	HIV	A03	1069.44
KMIGGIGGFIK	HIV	A03	1069.45
AIFQSSMTK	HIV	A03	966.01
WTYQIYQEPFK	HIV	A03	1069.46
FLGKIWPSHK*	HIV	A03	1069.56*
TVYYGVPVWK	ШΥ	A11	1052.03
VTVYYGVPVWK	HIV	A11	1069.47
GVAGALVAFK	HCV	A03	1073.10
CTCGSSDLY	HCV	A11	1069.62
GVAGALVAFK	HCV	All	1052.05
LLDTASALY*	HBV	A01	1069.01*
TLWKAGILYK	HBV	A03	1069.15
borderline positive			

. . . .

25

35

WHAT IS CLAIMED IS:

1. A method for activating cytotoxic T cells in vitro comprising:

dissociating bound peptides from class I MHC molecules
on antigen presenting cells, using a mild acid treatment;
associating desired immunogenic peptides with the
class I MHC molecules on the antigen presenting cells; and
incubating the antigen presenting cells with the
cytotoxic T cells in the presence of a growth factor, thereby
producing activated cytotoxic T cells.

- 2. The method of claim 1, wherein the step of dissociating bound peptides is carried out by incubating the antigen presenting cells in a glycine or citrate-phosphate buffer solution at pH 3.
 - 3. The method of claim 1, wherein the step of associating desired immunogenic peptides with the MHC molecules is carried out by incubating the antigen presenting cells with about 10 to 50µg/ml immunogenic peptide.
 - 4. The method of claim 1, wherein the step of incubating the antigen presenting cells with the cytotoxic T cells for about 7 to about 10 days.
 - 5. The method of claim 1 wherein the antigen presenting cells are peripheral blood mononuclear cells isolated from a patient.
- 30 6. The method of claim 5 wherein the peripheral blood mononuclear cells are SAC-I activated.
 - 7. The method of claim 1 wherein the incubating step includes a growth factor.
 - 8. The method of claim 8 wherein the growth factor is IL-7 and said growth factor is added at day 0 and day 7.

PCT/US94/08672

- 9. The method of claim 7 wherein the growth factor is IL-2 and said growth factor is added after day 7.
- 10. The method of claim 1, further comprising:
 5 contacting the activated cytotoxic T cells with an acceptable carrier, thereby forming a pharmaceutical composition; and administering the pharmaceutical composition to a patient.
- 11. The method of claim 10, further comprising 10 separating the activated cytotoxic T cells from the antigen presenting cells.
- 12. The method of claim 10 wherein the cytotoxic T cells are useful in the treatment of cancer, AIDS, hepatitis,15 bacterial infection, fungal infection, malaria or tuberculosis.
 - 13. A method of specifically killing target cells in a human patient, comprising:

obtaining a fluid sample containing cytotoxic T cells from the patient;

contacting the cytotoxic T cells with antigen presenting cells comprising class I MHC molecules having selected immunogenic peptides associated therewith, thereby producing activated cytotoxic T cells;

contacting the activated cytotoxic T cells with an acceptable carrier, thereby forming a pharmaceutical composition; and

administering the pharmaceutical composition to a patient.

30

25

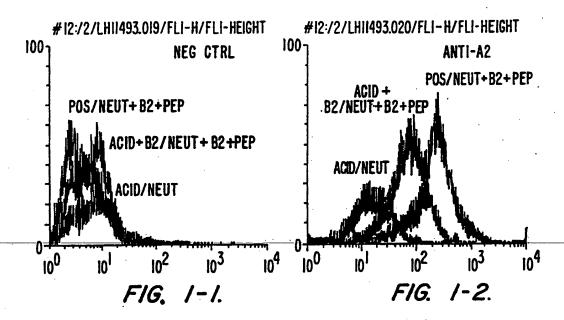
14. The method of claim 13, further comprising the step of dissociating bound peptides from the antigen presenting cells by incubating the antigen presenting cells in a glycine or citrate-phosphate buffer solution at pH 3.

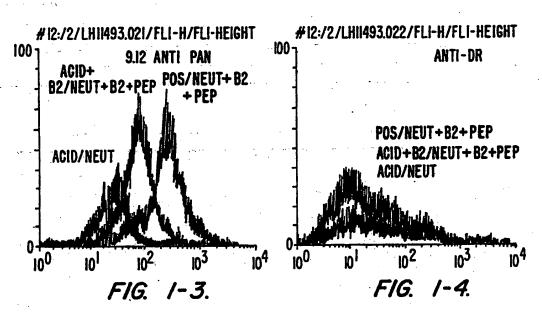
35

15. The method of claim 12, further comprising the step of associating desired immunogenic peptides with the MHC molecules on the antigen presenting cells by incubating the

antigen presenting cells with about 10 to $50\mu g/ml$ immunogenic peptide.

- 16. The method of claim 12 wherein the antigen5 presenting cells are peripheral blood mononuclear cells isolated from a patient.
- 17. The method of claim 12, wherein the step of incubating the antigen presenting cells with the cytotoxic T10 cells for about 7 to about 10 days.





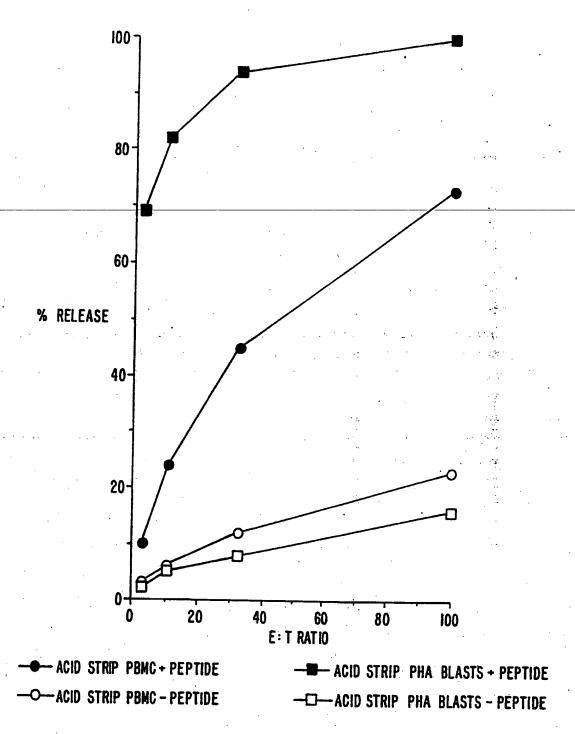
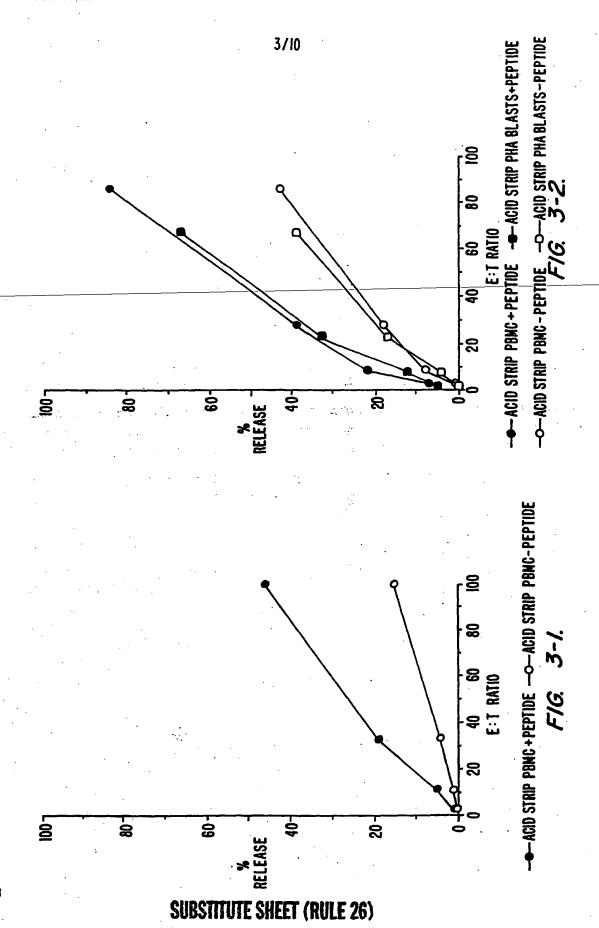


FIG. 2.

SUBSTITUTE SHEET (RULE 26)



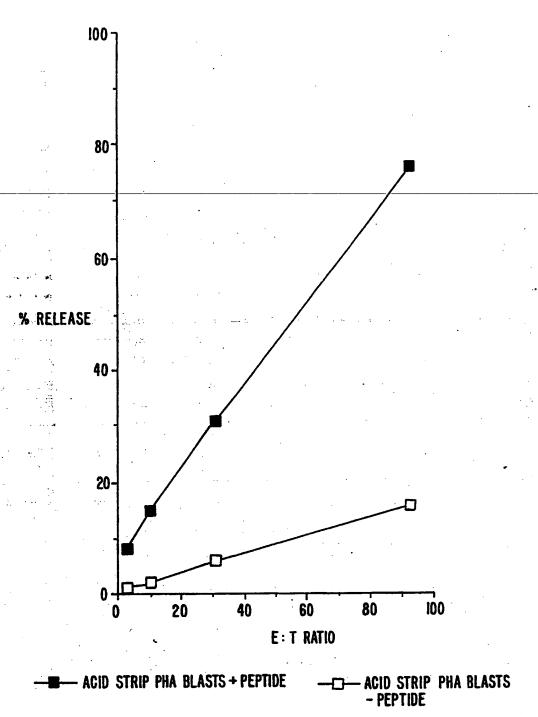
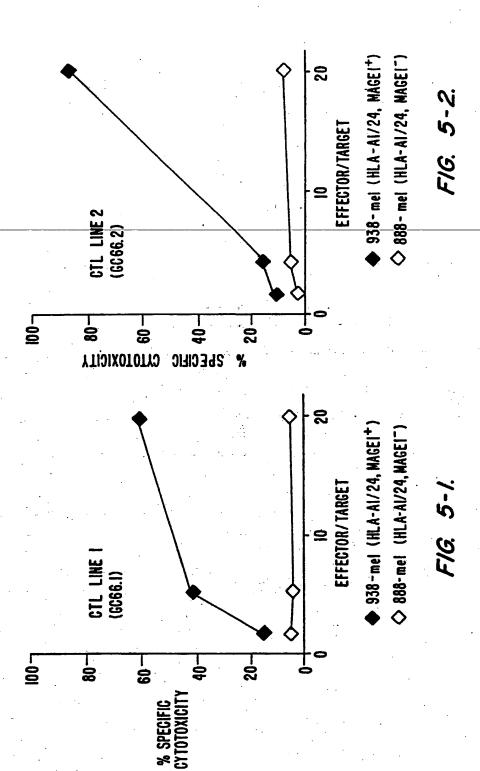
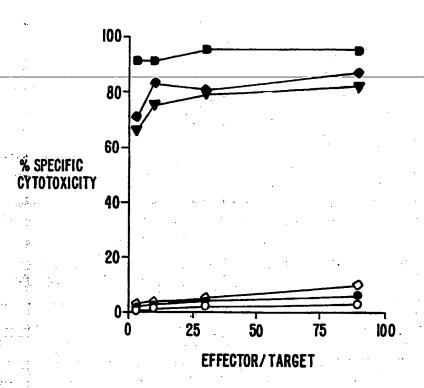


FIG. 4.

SUBSTITUTE SHEET (RULE 26)



SUBSTITUTE SHEET (RULE 26)



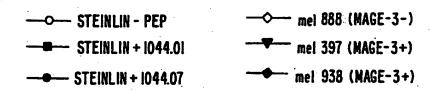
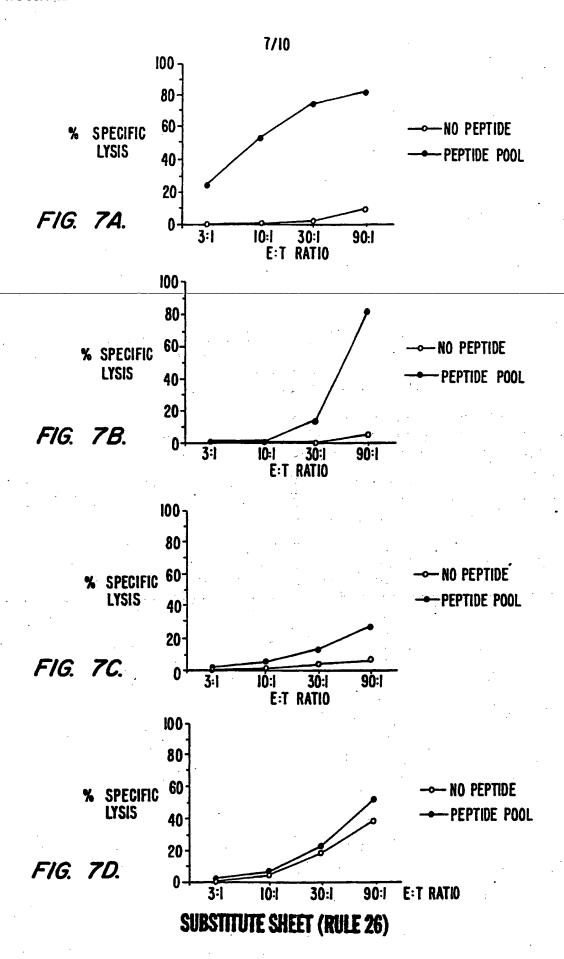
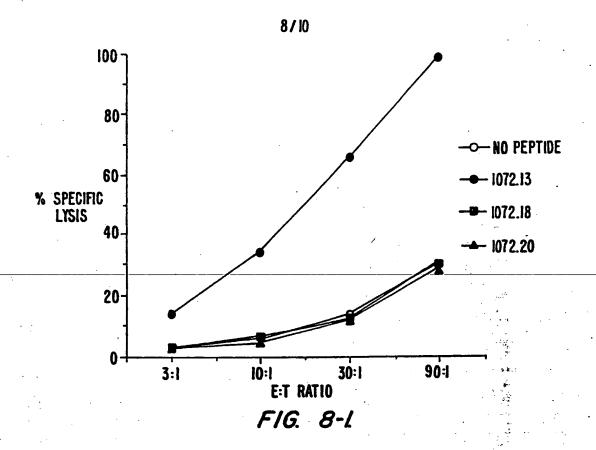
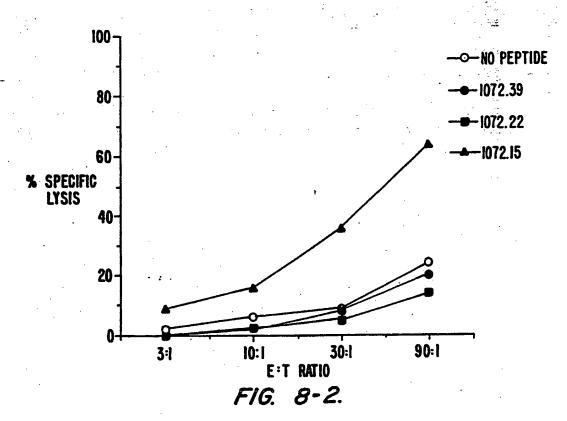


FIG. 6.

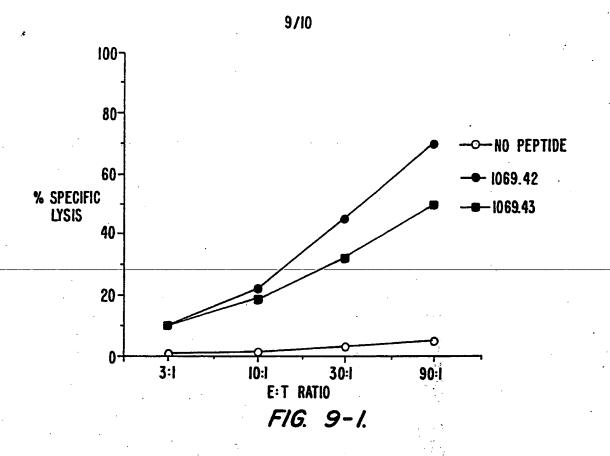


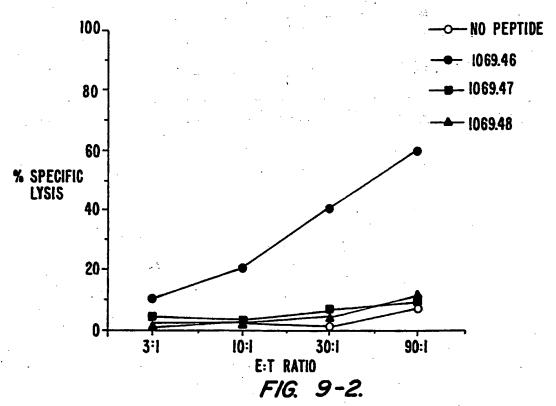
PCT/US94/08672





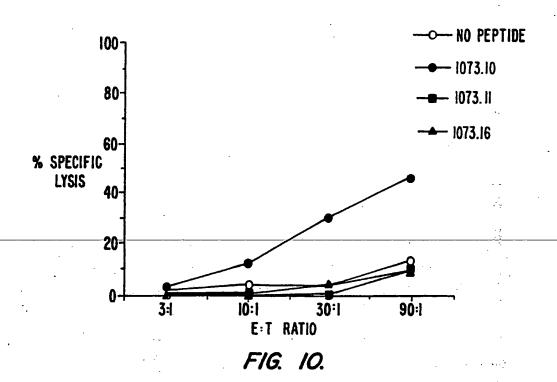
SUBSTITUTE SHEET (RULE 26)

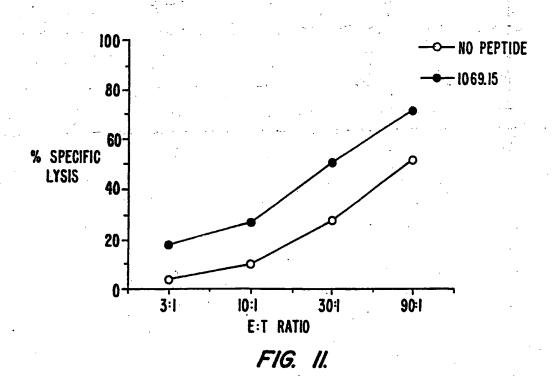




SUBSTITUTE SHEET (RULE 26)

10/10





SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No. PCT/US94/08672

A. CLASSIFICATION OF SUBJECT MATTER IPC(6) :C12N 5/08; A61K 35/14, 39/00 US CL :424/93.1, 93.7, 93.71; 435/240.1, 240.2				
	to International Patent Classification (IPC) or to both	national classification and IPC		
	LDS SEARCHED	1 by classification symbols)		
	documentation searched (classification system followed	by classification symbols)		
0.5. :	424/93.1, 93.7, 93.71; 435/240.1, 240.2			
Documenta	tion searched other than minimum documentation to the	e extent that such documents are included	in the fields searched	
Electronic o	data base consulted during the international search (na	ame of data base and, where practicable	, search terms used)	
APS; DIA	ALOG DATABASES: BIOSIS PREVIEWS, MEDLI	NE, AIDSLINE, WORLD PATENT IN	DEX, CA SEARCH	
C. DOC	CUMENTS CONSIDERED TO BE RELEVANT			
Category*	Citation of document, with indication, where ar	opropriate, of the relevant passages	Relevant to claim No.	
Y	US, A, 5,081,029 (ZARLING ET A entire document.	L.) 14 January 1992, see	1-17	
Υ	The Journal of Immunology, Volu	ima 146 issuad 01 Anril	1-17	
•	1991, Clerici et al., "Detection of		, , , , , , , , , , , , , , , , , , ,	
i	Specific for Synthetic Peptides of			
	Individuals," pages 2214-2219, se	—, •	. •	
Υ	Int. Conf. AIDS (Canada), 4-9 Jun	a 1999 Volume 5 issued	 1-17	
	1989, Koenig et al., "Determination		1-1:7	
	by HIV-specific CTL", page 531, abstract no. W.C.O. 41, see			
	entire abstract.			
A - 2.38	and the state of t			
	·			
Further documents are listed in the continuation of Box C. See patent family annex.				
• Sp	ocial categories of cited documents:	"T" later document published after the integrated and not in conflict with the applic		
A do	reument defining the general state of the art which is not considered be of particular relevance	principle or theory underlying the inv		
.E. car	riser document published on or after the international filing date	"X" document of particular relevance; the considered novel or cannot be considered.		
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other				
special reason (as specified) "Y" document of perticular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art				
P document published prior to the international filing date but later than *&* document member of the same patent family the priority date claimed				
Date of the actual completion of the international search Date of mailing of the international search report				
12 OCTOBER 1994 1 4NOV 1994				
Name and mailing address of the ISA/US Commissioner of Palents and Trademarks Box PCT Washington, D.C. 20231 Authorized officer JOHNNY F. RAILEY II. PH.D. 32				
	Box PCT Washington, D.C. 20231 JOHNNY F. RAILEY II, PH.D.			
Faceimile N	Fassimile No. (703) 305-3230 Telephone No. (703) 308-0196			